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MICROBIAL ENCAPSULATION AND INTERACTIONS IN EMULSIONS

by

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ABSTRACT

Emulsion-based formulations are composed of dispersed oil phase in water (oil-in-water (O/W) emulsions) or dispersed water phase in oil (water-in-oil (W/O) emulsions). In some emulsions, the dispersed phase comprises nano-sized droplets. Previous studies indicated that emulsions with nano-sized droplets or nano-emulsions possess anti-microbial activity by causing irreversible damage to cell membrane. This thesis investigated the effect of nano-emulsions on bacterial survival, growth, morphology and membrane's structural integrity as well as the effect of bacteria on the stability of the nano-emulsion. Exposure of bacterial cells to nano-emulsions was found to have no significant effect on the survival or growth bacteria and cell membrane integrity was not compromised. The morphology of bacteria was unchanged in nano-emulsion and cells remained in the water phase as single (planktonic) cells and the stability of the nano-emulsion was unaffected by the presence of bacteria.

The structure of emulsions can be complex. Double emulsions are formed by W/O emulsion dispersed in water (water-in-oil-in-water ($W_1/O/W_2$) emulsions). Such emulsion microstructures present interest for microbial encapsulation in food, cosmetic and pharmaceutical applications. Therefore microbe-emulsion interactions need to be characterised in order to fully explore the potential of such applications. This thesis investigated the stability of the $W_1/O/W_2$ emulsion in the presence of bacteria present in W_1 and W_2 phases as well as the mechanism of bacterial release from $W_1/O/W_2$ emulsion. $W_1/O/W_2$ emulsion preparation and bacterial cell encapsulation was achieved using a two-step homogenisation process. The

presence of bacteria affected the emulsion structure during the first step of the emulsification process but had no effect on emulsion structure during storage. Emulsion structure had no effect on bacterial viability while emulsions were stable in the presence of bacteria. For investigating bacterial release, the destabilisation of the $W_1/O/W_2$ emulsions was achieved by altering the osmotic pressure. The stability and release mechanism of bacteria from $W_1/O/W_2$ emulsion globules under hyper-osmotic and hypo-osmotic pressure was investigated as well as the impact of varying the amount of W_1 , hydrophilic surfactant (Tween80) in the outer aqueous (W_2) phase and altering the osmotic balance by adding salt (NaCl) in W_1 or W_2 phases. The release of bacteria occurred due to the bursting of the oil globules independent of diffusion mechanisms and be controlled by altering the structure of the $W_1/O/W_2$ emulsion. Finally, $W_1/O/W_2$ emulsion was investigated in real life application by incorporating in set-style yogurt model system. $W_1/O/W_2$ emulsion had a significant effect on the texture and physicochemical properties of the yogurt but no effect on bacterial growth kinetics. Also, probiotics were successfully incorporated in W_1 of $W_1/O/W_2$ emulsion and delivered in set-style yogurt showing high viability at the end of the acidification process.

In summary, this thesis demonstrates the feasibility and applicability of $W_1/O/W_2$ emulsion for the encapsulation of microbial cells for the purpose of their protection and triggered release. The results of this thesis can be used in the formulation of better probiotic products, segregation, protection and release of microbial cells during fermentation as well as for *in vivo* delivery of therapeutic cells.

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NOMENCLATURE

SYMBOLS

λ	Absorbance
atm	Atmospheres
d_i	Diameter of droplets
n_i	Number of droplets
n_D^{22}	Refractive index at 22 degrees Celsius
ϕ_i	The dispersed phase volume fraction
D (4,3)	Volume-weighted droplet diameter
ζ -potential	Zeta potential
η	Viscosity
K	Consistency coefficient
γ	Shear rate
n	Flow behaviour index

ABBREVIATIONS

BATH	Bacterial-adhesion-to-hydrocarbons
BSA	Bovine serum albumin

Ca	Calcium
CHD	Chlorhexidine diglucoate
CMC	Critical micelle concentration
Cryo-SEM	Cryogenic scanning electron microscopy
CPC	Cetylpyridinium chloride
CPI	Catastrophic phase inversion
CRI	Congo red indicator plates
CTAB	Cetyltrimethylammonium bromide
DAPI	2-(4-amidinophenyl)-1H-indole-6-carboxamide
DE	Double emulsion
Dex	Dextran
DiBAC ₄ (3)	Bis-(1,3-Dibutylbarbituric Acid)-trimethine-oxonol
DLS	Dynamic light scattering
EIP	Emulsion inversion point
FITC-BSA	Fluorescently tagged bovine serum albumin
<i>fps</i>	Frames per second
FSC	Forward scatter channel

FT-IR	Fourier transform infrared spectroscopy
GFP	Green fluorescent protein
HLB	Hydrophilic-liphophilic balance number
LED	Light emitting diode
O	Oil
MIB	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
M.R.S	<i>de Man, Rogosa and Sharpe</i>
NaCl	Sodium chloride
Na ₂ SO ₄	Sodium sulphate
PBS	Phosphate buffer saline
PI	Propidium iodide
pI	Isoelectric point
PIT	Phase inversion temperature
PGPR	Polyglycerol of polyricinooleates
QS	Quorum sensing
SC	Sodium caseinate

SDS	Sodium dodecyl sulfate
SE	Spontaneous emulsification
SEM	Scanning electron microscopy
SFC	Solid fat content
SLS	Static light scattering
SMP	Skimmed milk powder
SSC	Side scatter channel
TEM	Transmission electron microscopy
TGA	Thermo-gravimetric analysis
TPA	Texture profile analysis
TPI	Transitional phase inversion
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV	Ultraviolet
W	Water (subset 1 referring to internal encapsulated, subset 2 to external continuous aqueous phase)
VNBCs	Viable but non-culturable cells

CHAPTER 1: INTRODUCTION

1.1 Motivation

Emulsions are dispersions of two immiscible liquids such as oil and water, one (the dispersed phase) of which is dispersed as spherical droplets in the other (the continuous phase). A nano-emulsion is formed when the diameter of the droplets in emulsion is within the nano-scale. The physical properties of nano-emulsions can be quite different from coarse emulsions and therefore may influence growth and survival of microorganisms. It has been claimed that the nano-sized droplets in nano-emulsions possess high surface tension which can disrupt bacterial cell membrane (Hamouda *et al.*, 1999; Hamouda and Baker, 2000; Myc *et al.*, 2001; Hemmila *et al.*, 2010). However, there is evidence of contradiction and no consistency of effect on the same species of bacteria. The formation of nano-emulsions can be costly, energy and time consuming and therefore their antimicrobial activity needs to be established first. This thesis comprehensively investigated the effects of nano-emulsions on growth and survival of several bacterial species and highlighted the reasons behind the contradiction and in literature.

A more complex structure can exist known as double emulsion which is an emulsion within an emulsion. The most common type of double emulsion is the water-in-oil-in-water or $W_1/O/W_2$ emulsion which has gained interest due to its ability to entrap and protect various hydrophilic substances and control their release from W_1 to W_2 made them applicable in various fields such as food, pharmaceuticals, and cosmetics. The release of hydrophilic substances that are dissolved in the inner W_1 phase has been

extensively investigated, however, there is a lack in studies investigating how non-soluble particles such as bacteria can be released. In this thesis the mechanism responsible for release of bacteria from W_1 to W_2 was investigated and the effect of changing the structure of $W_1/O/W_2$ emulsion on the release mechanism was studied.

Yogurt is a widely consumed food associated with the consumption of probiotics which are added either as part of the starter culture or by fortification during the fermentation process (Lourens-Hattingh and Vijoen, 2001). Set-type yogurt is produced by milk acidification directly into containers without any further stirring aiming thicker texture than for most other yogurts. Thus, the challenge is to incorporate probiotic bacteria without compromising texture development. Furthermore, probiotic bacteria in yogurt need to remain viable and resist stresses i.e. resist manufacture processes (Rodríguez-Huezo *et al.*, 2014), storage at refrigeration temperature (Xin *et al.*, 2009), and digestion conditions (Shima *et al.*, 2006), in order to reach the gut in functional concentrations ($>10^8 \log_{10} \text{CFU/g}$) (Kechagia *et al.* 2013). However, probiotic bacteria can interfere with starter cultures and compete during fermentation (Vinderola *et al.*, 2001) and/or alter fermentation and quality of final product. Therefore, encapsulation of probiotic bacteria could increase their viability in foods and post-digestion as well as enabling the spatial separation of strains that negatively affect yogurt fermentation but present beneficial effects post-consumption.

Micro-encapsulation has been used as a solution for some of these problems. For example, various polymers (e.g. alginate) have been used to encapsulate and protect probiotics from storage at low temperature and gastrointestinal conditions (Sultana *et al.*, 2000). However, polymer based encapsulation technologies are not compatible

with dairy-based foods such as yogurt and $W_1/O/W_2$ emulsions could be a more suitable alternative as they can be made from ingredients that are compatible with yogurt. Furthermore, for encapsulation of probiotics, $W_1/O/W_2$ emulsion is superior in comparison to edible polymers as they provide extended benefits including fat and salt reduction and release aroma and flavour while maintaining the taste perception.

The encapsulation of microbial species in $W_1/O/W_2$ emulsion has been investigated for various applications. The encapsulation of *Lactobacillus* spp. probiotics in $W_1/O/W_2$ emulsions have shown to protect from cytotoxic gastric juice (Shima *et al.*, 2006; Pimentel-González *et al.*, 2009) bile salts, (Shima *et al.*, 2009), prolonged storage at low temperatures and during cheese manufacturing (Rodríguez-Huezo *et al.*, 2014) and melting (Xin *et al.*, 2009). However, studies on the ability of $W_1/O/W_2$ emulsion to protect probiotics during fermentation, storage and digestion of yogurt is lacking. In this thesis the survival of encapsulated probiotic *Lactobacillus paracasei* during fermentation, storage and digestion of set-type yogurt was assessed and the stability of the system over storage was investigated by monitoring bacterial survival, physicochemical and textural properties.

1.2 Aims of this research

The overall aim of this project was to study the effect of O/W nano-emulsion on survival and growth of bacteria and investigate the release mechanism of bacteria from $W_1/O/W_2$ emulsion as well as the feasibility of $W_1/O/W_2$ emulsion for delivery of probiotics in set-type yogurt. The objectives can be summarised as:

- Working with components that are “food grade” and commonly used in the food industry;

- Investigate the effect of O/W nano-emulsions on the survival and growth of bacteria; Study the stability of $W_1/O/W_2$ emulsion in the presence of bacteria during storage;
- Study the stability of $W_1/O/W_2$ emulsion in the presence of two different bacterial species present within the W_1 and/or W_2 phase;
- Characterise the mechanism of bacterial release in $W_1/O/W_2$ emulsion, the effect of the amount of hydrophilic surfactant and W_1 phase and altering the osmotic balance in W_1 or W_2 phase on the release of bacteria from $W_1/O/W_2$ emulsion;
- Investigate the incorporation of $W_1/O/W_2$ emulsion and encapsulated probiotic bacteria in a set-type yogurt.

1.3 Outline

This manuscript is composed of eight chapters: an introduction, five results chapters, conclusion and future work, appendix, and references.

- Chapter 2 is the literature review that provide a summarised review of literature relevant to subjects mentioned in this thesis;
- Chapter 3 is the first result chapter, regarding the effect of nano-emulsion on bacterial survival and growth and the effect of bacteria on stability of the emulsion;
- Chapter 4 is the second result chapter investigating the stability of $W_1/O/W_2$ emulsion in the presence of bacteria in the inner W_1 and outer W_2 phase;

- Chapter 5 is the third result chapter in which the mechanism of release of bacteria from $W_1/O/W_2$ emulsion was studied and the effect of changing the amount of hydrophilic surfactant and W_1 phase on this release under hyper-osmotic pressure was investigated;
- Chapter 6 is the fourth result chapter in which the effect of changing the amount of hydrophilic surfactant and W_1 phase on the release of bacteria from $W_1/O/W_2$ emulsion under hypo-osmotic pressure was investigated;
- Chapter 7 is the fifth and final results chapter in which the attempt to incorporate $W_1/O/W_2$ emulsion hosting probiotic bacteria in set-type yogurt and investigate the physicochemical changes that occur during the acidification process and survival of the probiotic was investigated;
- Chapter 8 is a summary of conclusion made throughout this study;
- Chapter 9 is suggested future work;
- References;
- Appendices.

1.4 Dessimation

Oral presentations

- H. El Kadri, T. Overton, S. Bakalis and K. Gkatzionis, *Understanding and controlling the release mechanism of Escherichia coli in double $W_1/O/W_2$ emulsions*, EFFOST conference, Athens, 2016.
- H. El Kadri, G. Ramazan, T. W. Overton, S. Bakalis and K. Gkatzionis, *Modulating the release of bacteria from water-in-oil-in-water ($W_1/O/W_2$) emulsions under hypo-osmotic pressure*, EFFOST conference, Vienna, 2017.

Poster presentations

- H. El Kadri, G. Ramazan, T. W. Overton, Mills, T. and K. Gkatzionis, Effect of structure of emulsions on *Escherichia coli*, SfAM summer conference, Brighton, 2014.
- H. El Kadri, T. W. Overton, and K. Gkatzionis, Controlling bacterial release from water-in-oil-in-water ($W_1/O/W_2$) emulsions, SfAM summer conference, Dublin, 2015.

Publications

- El Kadri, H., Devanthi, P. V. P., Overton, T. W. and Gkatzionis, K. 2017. Do oil-in-water (O/W) nano-emulsions have an effect on survival and growth of bacteria? *Food Research International*. **101**,114-128, DOI:10.1016/j.foodres.2017.08.064
- El Kadri, H., Overton, T. W., Bakalis, S. and Gkatzionis, K. 2015. Understanding and controlling the release mechanism of *Escherichia coli* in double $W_1/O/W_2$ emulsion globules in the presence of NaCl in the W_2 phase, *RSC Advances*, **5**, 105098-105110. DOI:10.1039/C5RA24469B
- El Kadri, H., Ramazan, G., Overton, T. W., Bakalis, S. and Gkatzionis, K. 2016. Modulating the release of *Escherichia coli* in double $W_1/O/W_2$ emulsion globules under hypo-osmotic pressure, *RSC Advances*, **6**, 93694-93706. DOI:10.1039/C6RA17091A

CHAPTER 2: LITERATURE REVIEW

2.1 Types of emulsions

2.1.1 Single and multiple emulsions

Emulsions are dispersions of two immiscible liquids such as oil and water, one (the dispersed phase) of which is dispersed as spherical droplets in the other (the continuous phase). The formation of emulsion requires emulsifiers which are molecules that stabilise the emulsion by increasing the repulsion between the dispersed phases. According to the distribution of liquid phases the emulsions can be classified. A simple emulsion that consists of oil globules dispersed in an aqueous continuous phase is known as oil-in-water or O/W emulsion (e.g. cream, milk, and mayonnaise) (Figure 2.1A). A simple emulsion that consists of water droplets dispersed in an oily continuous phase is known as water-in-oil or W/O emulsion (e.g. margarine, butter and chocolate) (Figure 2.1B) (McClements, 2004). The emulsion formed is determined by the type of emulsifier which can either be hydrophilic forming an O/W emulsion or lipophilic forming a W/O emulsion. Types of emulsifiers will be discussed in more detail in Section 2.3.

A more complex structure can exist such as multiple or double emulsion, a structure in which the droplets of the dispersed phase entrap smaller droplets of similar (but not necessarily identical) composition as the continuous phase. The two most common double emulsions include the water-in-oil-in-water or $W_1/O/W_2$ in which an oily phase separates an inner water (W_1) phase and an outer water (W_2) phase (Figure 2.1C) and the oil-in-water-in-oil emulsion in which a water phase separates an inner oily (O_1) phase and an outer (O_2) phase (Figure 2.1D) (Dickinson and

McClements, 1995). These double emulsions are advantageous over simple emulsions in certain applications such as reducing the caloric value of food emulsions while maintaining their organoleptic quality and carrying, protecting, or controlling the release of active ingredients (Norton and Norton, 2010; Garti and Bisperink, 1998). Higher-order multiple emulsions exist where the triple ($O/W/O/W$ or $W/O/W/O$) and quadruple emulsions ($W/O/W/O/W$ or $O/W/O/W/O$) are formed but these are still in their early research (Vladisavljević *et al.*, 2017).

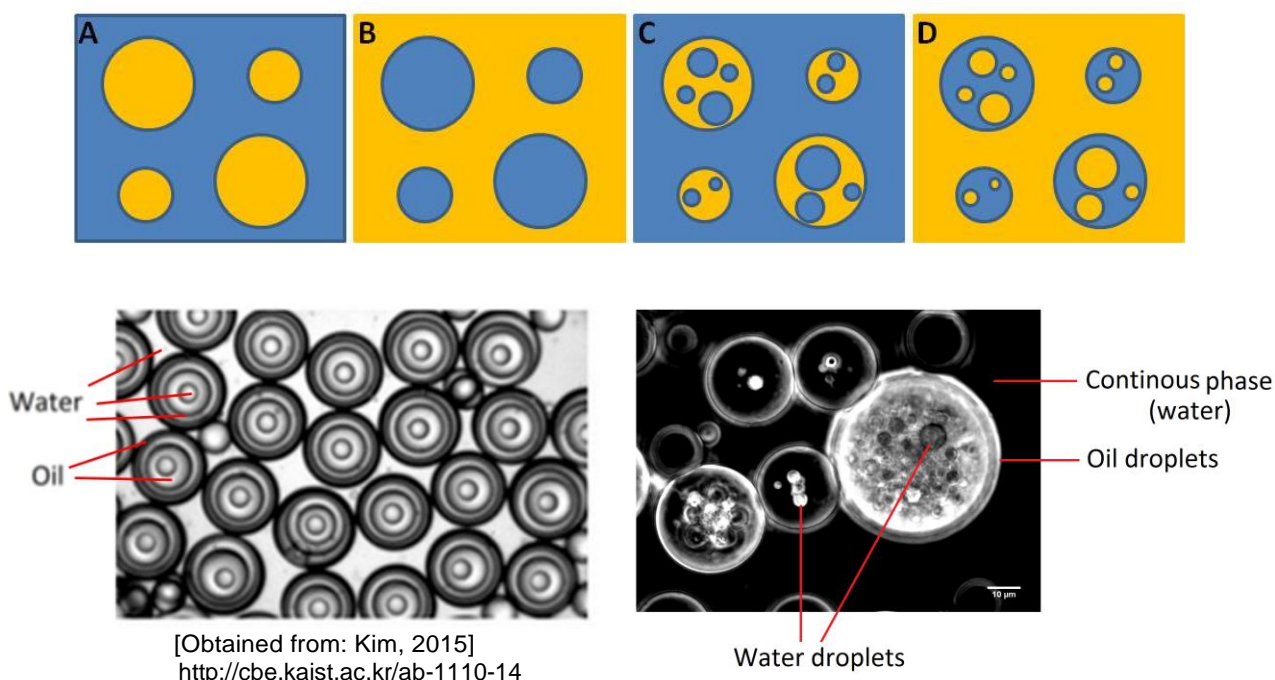


Figure 2.1 Schematic representation of emulsion types. O/W emulsion (A) W/O emulsion (B) $W_1/O/W_2$ emulsion (C) and $O_1/W/O_2$ emulsion (D) (blue and yellow represent water and oil phases respectively). Real microscopic images of a quadruple $W/O/W/O/W$ (E) and double $W_1/O/W_2$ emulsion (F).

2.1.2 Coarse, nano- and micro-emulsions

Emulsions can be classified according to the size of their droplets. In general droplets in food emulsions are in the micro-meter range (droplet diameter $>1\mu\text{m}$) and these are referred to as coarse emulsions throughout this thesis. However, a type of

emulsion known as a nano-emulsion contains droplets within the nanometre range. There is controversy in the literature regarding diameter size of the droplets in nano-emulsions and they range from 20-800nm (Tadros *et al.*, 2004; McClements and Rao, 2004; Anton *et al.*, 2008; Gupta *et al.*, 2016). For emulsions to be able to form they require an input of energy by applying shear force (McClements, 2012a) and they tend to be thermodynamically unstable since droplets merge into each other upon collision causing the two phases to separate. Coarse emulsions are considered to be thermodynamically and kinetically unstable whereas nano-emulsions are considered to be thermodynamically unstable and kinetically stable (Gupta *et al.*, 2016). When the droplet size is reduced the characteristics and appearance of the emulsion tend to change as well. For, example coarse emulsions would have creamy or whitish appearance whereas emulsions with droplet size less than 30nm tend to be translucent or transparent.

Another form of emulsion exist known as micro-emulsion with diameter of droplet size ranging from 2-100nm and in contrast to the other emulsions they are considered to be thermodynamically stable (Dalgleish, 2006; McClements and Rao, 2004; Gupta *et al.*, 2016). These are formed by thermodynamic molecular self-assembly of emulsifier without the need for shear force and are formed with low energy input. Micro-emulsions are not going to be discussed here as they are beyond the scope of the thesis.

2.2 Droplet characteristics

The presence of the dispersed phase as droplets plays an important role in determining the physicochemical and sensory properties of food emulsions. The important characteristics of emulsion droplets are discussed below.

2.2.1 Droplet concentration

The concentration of droplets can determine the stability, organoleptic and bulk physicochemical properties of food emulsions. The dispersed phase 'volume fraction' (ϕ_i) is a term that describes the concentration of the droplets and is defined as the volume of the dispersed phase divided by the volume of all the constituents of the emulsion prior to mixing. The dispersed volume fraction is an important factor that can affect the choice of the relative number of ingredients and process conditions (McClements, 2004).

2.2.2 Droplet size distribution

The size of the droplets can determine many of the properties of food emulsions (e.g. appearance, texture and stability) (Dickinson, 1992). Monitoring the droplet size allows for studying the stability of the emulsions and understanding their behaviour during storage conditions. Emulsions with droplets that are all of one size are referred to as monodisperse while emulsions with a range of droplet sizes are referred to as polydisperse. In monodisperse emulsions the droplet size is reported as a single number. However, the industry encounters polydisperse emulsions and the size of the droplets is reported as a distribution of droplet sizes. In most cases information on the mean or average size of the droplets and the width of the

distribution will suffice (Hunter, 1986). The droplet size distribution is depicted using a histogram of volume frequency of known size-class of droplets (Silva *et al.*, 2013). Depending on how the distribution data are collected and analysed the mean droplet size in units of 'length' can be calculated. One of the most useful common mean droplet sizes is referred to as the De Broucker (or volume) mean droplet size [D (4, 3)] and is defined according to:

$$D [4,3] = \frac{\sum n_i d_i^4}{\sum n_i d_i^3} \quad (2.1)$$

where n_i is the number of droplets with diameter d_i . The D (4, 3) is used when the aim is to measure the size of the coarse droplets that make up the bulk of the emulsion.

2.2.3 Droplet interfacial properties

The narrow region that separates between the dispersed and continuous phase is known as the interface (Figure 2.2). The interface is the region that determines the physicochemical properties of the emulsion (Hunter, 1986). Emulsion stability can be altered by changing the factors that affect the rheology, thickness and charge of the interfacial region. The characteristics of the interfacial region are determined by the type and concentration of emulsifier or surfactant and other surface-active molecules that adsorb at the interface as well as events that take place at the time of and after emulsion formation (McClements, 2004). By controlling the interfacial properties, it is possible to obtain an emulsion with desired functional properties.

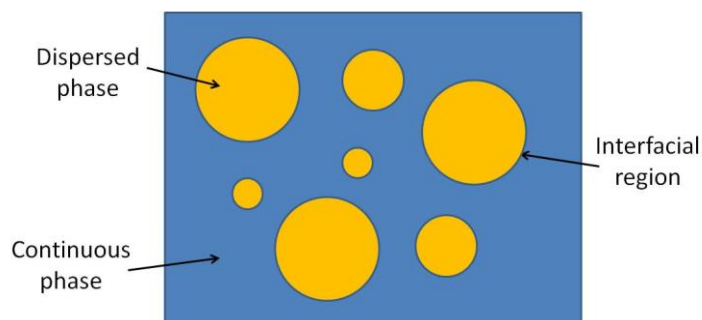


Figure 2.2 Interfacial region separating the dispersed and continuous phases.

2.2.4 Droplet charge

The droplets of an emulsion possess an electrical charge as a result of adsorption of ionic species at the interface. The electrical charge of emulsion droplets affects the stability of the emulsion. The type and concentration of adsorbed emulsifier and surface-active molecules at the interface will determine the magnitude and sign of the electrical charge (Grumezescu, 2016). Ionic species of opposite charge will attract each other while the ones of similar charge will repel each other. In emulsions, all the droplets will contain the same electrical charge as they are stabilised by the same emulsifier molecules and exposed to the same environment. When proteins are adsorbed at the interface both steric and electrostatic repulsions can be induced, and the droplet charge depends on the pH of the continuous phase relative to the isoelectric point (pI) of the protein, a pH value at which the protein has zero charge (Gu *et al.*, 2005). When polysaccharides are adsorbed at the interface electrostatic repulsion is induced and the droplet charge is negative. Ionic surfactants induce electrostatic repulsions with negatively (anionic surfactants) or positively (cationic surfactants) charged droplets. Non-ionic surfactants such as Tween80 will exert steric repulsion due to their polyoxythelene chains and droplets will have no charge.

However, interfaces stabilised by Tweens fit well with the model of electrostatic repulsion and in the presence of salts the droplets will have a slightly negative charge (Logan *et al.*, 2013). The ionic properties of droplet in emulsion can be described in terms of its zeta potential (ζ -potential) which is a measure of surface charge of the droplet (Hunter, 1986) which will be later discussed. By choosing emulsifiers that possess desirable charge characteristics and controlling the properties of the continuous phase it is possible to manipulate the charge of the droplets in emulsion.

2.2.5 Droplet crystallinity

In any fat-containing microstructure the percentage of total fat which is crystallised or solidified at a particular temperature is known as the solid fat content (SFC). Depending on the SFC the fat can vary from being completely solid (at low temperatures) to being completely liquid (at high temperatures) (McClements, 2004). The crystallisation of fat plays an important role in the physicochemical and organoleptic properties of food emulsions (Dickinson and McClements, 1995). The preparation of emulsions requires that the oil phase remains liquid during the homogenisation process (McClements, 2012b). However, when using fat as the dispersed phase, the homogenisation process is usually done by melting the fat and then dispersing it in a pre-heated aqueous phase containing the emulsifier to form the emulsion. Once the emulsion is cooled down to a certain temperature that is below the melting point a suspension of crystals dispersed in water will form (Coupland, 2002). The process of crystallisation requires the formation of a crystalline phase (nucleation) and then subsequent crystal growth. When the oil phase contains no impurities, it will remain as a liquid down to a temperature at which nucleation occurs (i.e. homogenous nucleation). Impurities in the oil phase can

initiate crystallisation acting as the starting point for nucleation down to a temperature where the process will occur more rapidly (i.e. heterogeneous nucleation) (McClements, 2012b). The number of droplets in an emulsion is significantly higher than the amount of impurities and therefore the majority of droplets are free of impurities and hence crystallise by an apparently homogenous mechanism (Coupland, 2002). The size of droplets in an emulsion is known to impact crystallisation properties of the dispersed phase. When the droplet size is reduced the crystallisation temperature becomes lower. In emulsion systems, the presence of emulsifiers plays a role in crystallisation behaviour. Emulsifier-induced heterogeneous nucleation can only be initiated when the emulsifiers are of similar molecular structure to that of the dispersed phase. For example, the hydrophobic tail groups of Tween80 act as a template for initiating surface heterogeneous nucleation while proteins are unable to initiate surface heterogeneous nucleation due to their chemical structure being incompatible with the triglycerides mix (Truong *et al.*, 2014).

2.3 Emulsifiers

The formation of an emulsion requires an input of energy and this can be done by homogenisation. Emulsions are thermodynamically unstable and the contact between the oil and water molecules is energetically unfavourable therefore the collision of the droplets with each other cause them to merge or coalesce leading to complete phase separation (McClements, 2004).

The differences in attractive interactions between molecules of oil and water will result in the interfacial tension between the two phases which is the force that holds the surface of a particular phase together. The interfacial tension acts to minimise the

interfacial area between the two phases and therefore reducing the interfacial tension will minimise the effects of interfacial forces and kinetically impede the coalescence of the droplets (Israelachvili, 1994). Emulsifiers are small amphiphilic molecules that reduce the interfacial tension between the oil and water phase. These molecules adsorb at the O/W interface and prevent them from coalescing. The description of emulsifiers in this section is focused on the ones used in this study.

2.3.1 Hydrophile-lipophile balance

The hydrophile-lipophile balance (HLB) is a method used to classify emulsifiers. This method generates a value (ranging from 1-20) which indicates whether the emulsifier is more soluble in oil or water. When no emulsifier is used the type of emulsion that forms depends on the volume fraction of each phase and the phase with lower volume will be the dispersed phase. However, in the presence of emulsifiers and according to the Bancroft rule the emulsifier used will determine what type of emulsion will form regardless of volume fraction of the phases. For example, low HLB emulsifiers (<6) will form a W/O emulsion while high HLB emulsifiers (>8) will form an O/W emulsion (McClements, 2004).

2.3.2 Surfactant stabilised emulsions

Surfactants are amphiphilic molecules, meaning that each surfactant molecule possesses a hydrophilic group (head part) and a hydrophobic group (tail part) (Figure 2.3). The head part of the surfactant molecule can be non-ionic, anionic, cationic or zwittertonic. The head part contains no charge in non-ionic surfactants, is negatively charged in anionic surfactants, is positively charged in cationic surfactants (e.g. lauric arginate) and bears both charges in zwittertonic surfactants. The tail part of the

surfactant molecule is made up of one or more non-polar hydrocarbon chains. Once a surfactant molecule is adsorbed at the interface the head part will be in the water phase while the tail part will reside in the oil phase. The surfactants used in this study have head parts that are non-ionic (McClements, 2004).

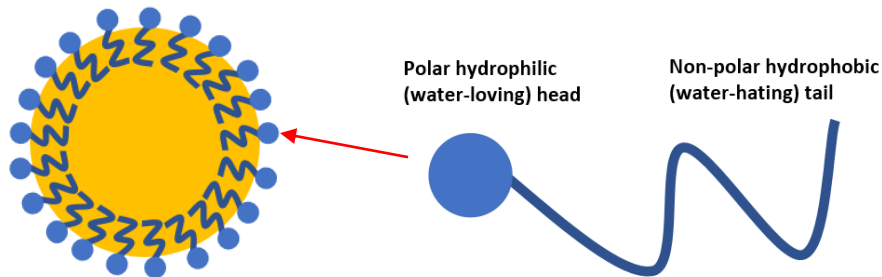


Figure 2.3 Surfactant molecule with polar hydrophilic (water-loving) head and non-polar hydrophobic (water-hating) tail.

During emulsification of oil and water and break up of droplets the surfactant molecules orient at the interface forming surface or interfacial films that prevent droplet coalescence. When two droplets come close to each other and collide the continuous phase squeezes out between droplets, dragging some of the surfactants. This creates a concentration gradient and more surfactant molecules adsorb at the O/W interface of the two droplets moving from regions of higher to lower surfactant concentration dragging some of the continuous phase along with them leading to an increase in the film thickness. This will cause water to move in the direction of highest interfacial tension and as a result the droplets move apart to prevent coalescence, this phenomenon is known as the Gibbs-Marangoni effect (Wilde *et al.*, 2004; Walstra, 1993).

When added to water only, surfactant will remain as isolated molecules and above a certain concentration, known as the critical micelle concentration (CMC), where further addition of surfactant will lead to the molecules to arrange as spherical micelles - thermodynamically stable structures. Only above the CMC the surfactant molecules can act as emulsifiers. With increasing surfactant concentration above CMC various micelle arrangements can be observed starting with spherical micelles, then rods of cylindrical micelles and hexagonal followed by cubic arrangements of cylindrical micelles and as more surfactant is added the formation of a lamellar phase (McClements, 2004).

2.3.3 Non-ionic surfactants

Non-ionic surfactants are the most common surfactants encountered in the food industry. The use of non-ionic surfactants is preferred over ionic surfactants because they are biodegradable and can cover a wide range of HLB values. In non-ionic surfactants, the hydrophobic part is fatty acid triglycerides that can be obtained from animal or plant sources. Most non-ionic surfactants are derived from the higher members of the series such as palmitic, steric, oleic, and linoleic acid (McClements, 2004).

2.3.4 Sorbitan esters and polysorbates (Tweens)

Sorbitan esters of fatty acids (also known as Spans) are non-ionic surfactants that are used in the food industry. They are low HLB lipophilic emulsifiers that stabilise W/O emulsions. Sorbitan esters of fatty acids are waxy solids or viscous liquids derived from a reaction of sorbitol and a commercial grade fatty acid (usually a mixture, e.g. stearic and oleic). Sorbitol is produced from hydrogenation of glucose

and it belongs to a group of compounds called sugar alcohols or polyols. Sorbitan esters are produced by direct fatty acid esterification of sorbitol with fatty acids (Brown, 1943; Kubie *et al.*, 1963).

Sorbitan esters can be further modified into polyoxyethylene sorbitan esters or polysorbates (also known as Tweens). The sorbitan esters are further reacted with ethylene oxide (derived from petrochemical industry) in the presence of small amounts of a basic catalyst (e.g. potassium hydroxide) to produce the polyoxyethylene sorbitan esters or polysorbates. Polysorbates are non-ionic, high HLB hydrophilic surfactants that stabilise O/W emulsions. They are powerful steric stabilisers where the bulky hydrated polyoxyethylene chains of polysorbate repel each other. The most common types of Tween include:

- Tween 20 or polyoxyethylene (20) sorbitan monolaurate;
- Tween 40 or polyoxyethylene (20) sorbitan monopalmitate;
- Tween 60 or polyoxyethylene (20) sorbitan monostearate;
- Tween 80 or polyoxyethylene (20) sorbitan monooleate.

The number 20 in brackets refers to the total number of oxyethylene units - (CH₂CH₂O)- per molecule and the number following Tween (20, 40, 60 or 80) corresponds to the type of fatty acid associated with the polyoxyethylene sorbitan part of the molecule.

Polysorbate80 or Tween80 is commonly used in food manufacturing. It is a viscous yellow liquid derived from polyethoxyethylated sorbitan and oleic acid. It is used in bakery, dairy and ice cream. In ice cream, Tween80 is added up to 0.5% (v/v) to increase smoothness and make handling easier as well as increase resistance to

melting (Goff, 1997). Polysorbate80 have been used safely in food products and EU regulations permit the use of polysorbates including Tween80 and the acceptable daily intake is less than 25 mg/kg per body weight (Gates, 2010).

2.3.5 Polyglycerol polyricinoleate (PGPR)

Polyglycerol polyricinoleate (PGPR) is a polyglycerol ester of castor oil fatty acid used in the food industry. It is a non-ionic, low HLB lipophilic surfactant that stabilises W/O emulsions and provides stability by steric repulsion. It is made up of short chain glycerol molecules connected by ether bonds with ricinoleic acid side chains connected by ester bonds. The synthesis of polyglycerol involves a dehydration process where both inter- and intra-etherification takes place. Ricinoleic acid or polyricinoleate is a hydroxy- and mono-unsaturated fatty acid derived from castor bean oil or just castor oil of the plant *Ricinus Communis L.* of the family *Euphorbiaceae*. Synthesis of PGPR is a direct esterification between polyglycerol and polyricinoleate in the presence of a basic or an acidic catalyst. PGPR is yellow to light brown, highly viscous liquid used in chocolates, spreadable fats, creamers, dairy analogues and a few other food emulsions. In the EU, it is listed by the reference number E476. In EU regulations, the accepted level of PGPR in food is 0.4% by weight. The acceptable daily intake is 4g/ kg of body weight for low fat spreads and dressings and 5g/kg of body weight for chocolates (Manufacturing confectioner, 1999). It has been shown that PGPR consumption has no adverse effect on health. However, since PGPR is of synthetic origin and has low limit of acceptable daily intake, there has been a lot of research trying to reduce or replace PGPR in food (Dickinson, 2011).

2.3.6 Proteins based emulsifiers

Proteins are amphiphilic molecules which are an essential component of the human diet. They are the most commonly used emulsifiers in the food industry. Various types of proteins are used in emulsion-based foods which can be derived from animal (O'Regan *et al.*, 2009; Anton *et al.*, 2009) and plant (Gonzalez-Perez and Arellano, 2009) sources. Due to their physicochemical properties proteins are not classified as surfactants. However, they possess polar and non-polar segments in their structure which giving them surface active properties. Proteins mainly provide stability against coalescence by steric and electrostatic (if electrically charged) repulsion (Dickinson and Gelin, 1992). The latter is influenced by pH and ionic strength of the continuous phase. When the pH reaches a value near the pI of the protein or when the ionic strength is high, electrostatic repulsion is reduced therefore increasing the chance of coalescence between the droplets (Dickinson and McClements, 1995).

Proteins also stabilise emulsions by adsorbing at the interface and form a visco-elastic interfacial film to prevent the droplets from coalescing (Wilde *et al.*, 2004). The conformational structure of proteins can be categorised into three types including rod-like (e.g. gelatine), random coil (e.g. casein) and globular (e.g. whey protein) (Dickinson, 2001). Globular and rod-like conformational structures are considered to be rigid, whereas random coil conformational structure has highly dynamic structures. After adsorbing at the interface random-coil proteins form a structure with the non-polar segments (loops) protruding in the oil phase while the polar segments (tails) protruding in the water phase whereas the neutral segments remain in direct contact with the oil and water interface (trains). Globular proteins on the other hand undergo

re-conformation at the interface with their non-polar segments facing the oil phase while their polar segments remain in the water phase. In comparison to random-coiled proteins, globular proteins tend to form highly visco-elastic interfacial films which are more resistant to rupture (Dickinson and McClements, 1995; Dickinson, 2001).

Milk contains proteins that exhibit good surface-active properties and provide stability against coalescence by both electrostatic and steric repulsion. Caseins make up the majority of the protein in milk and the remaining is whey protein. During emulsification casein molecules adsorb rapidly at the interface. In milk 75% of casein consists of α_{s1} -casein and β -casein (Swaisgood, 1982). However, β -casein provides a better surface coverage resulting in thicker interfacial films compared to α_{s1} -casein. Competitive adsorption occurs between caseins and β -casein will displace α_{s1} -casein at the interface (Brooksbank *et al.*, 1993). The main fractions which contribute to emulsifying properties of whey proteins are β -lactaglobulin and α -lactalbumin and these constitute about 70-80% of whey protein (Britten and Giroux, 1991). Compared to caseins whey molecules adsorb much slower at the O/W interface during emulsification and although they provide stability against coalescence, however, caseins have proven to be the better stabilisers of emulsions (Hunt and Dalgleish, 1994). Unlike caseins no competitive adsorption occurs between β -lactaglobulin and α -lactalbumin at the O/W interface. Whey protein also contains a small amount of lactoferrin, a glycoprotein that shows high surface coverage at O/W interface. Unlike β -lactaglobulin and caseins, emulsions stabilised with lactoferrin are stable over a wide range of pH. Lactoferrin is positively charged at neutral pH forming cationic

droplets whereas β -lactoglobulin, α -lactalbumin and casein proteins are negatively charged at neutral pH forming anionic droplets (Ye and Singh, 2006).

2.4 Emulsion stability

There are different mechanisms that can contribute to emulsion instability including gravitational separation, flocculation, Ostwald ripening, coalescence, and phase inversion. The mechanisms which contribute to the instability of single emulsions are common to double emulsions, however, the latter present greater instability due to higher structure complexity (Dickinson and McClements, 1996). In this section, the main mechanisms that lead to O/W and $W_1/O/W_2$ emulsion instability and the ways to improve it will be discussed.

2.4.1 Instabilities in O/W emulsions

Due to differences in the density of the dispersed and continuous phases of a thermodynamically unstable emulsion, the droplets can move up or down under the influence of gravity leading to phase separation where either a cream or a sediment layer appears (Lobo *et al.*, 2002). Creaming occurs when the dispersed phase has lower density than the continuous phase and the droplets will move upwards forming a cream layer at the top of the emulsion (Figure 2.4A). Sedimentation occurs when the dispersed phase has higher density than the continuous phase and the droplets will move downwards forming a sediment layer at the bottom of the emulsion (Figure 2.4B). In general, creaming is more prevalent in O/W emulsions while sedimentation is more prevalent in W/O emulsions. Gravitational separation can be quantified by measuring the velocity by which the emulsion separates (McClements, 2004). The velocity by which gravitational separation occurs depends on the density difference

between the liquids that make up the dispersed and continuous phases, the viscosity of the continuous phase and the droplet size. Various ways can be used to make the emulsion more resistant to gravitational separation including:

- Dispersed and continuous phases with similar densities are more resistant to creaming (Garti and Aserin, 1996). In O/W matching the densities of the two phases can be achieved by adding lipophilic weighting agents (e.g. ester gum) to the oil phase prior to the homogenisation process. This increases the density of the oil phase so that it could match the density of the continuous phase. Another way to increase the density of the dispersed phase is by stabilising the emulsion using highly dense emulsifiers.
- Smaller droplets are more resistant to creaming (Dickinson *et al.*, 1991). Decreasing the size of the droplets can be achieved by adding more emulsifier and increasing the amount of energy supplied during homogenisation (e.g. increasing the speed and time of rotational speed).
- Increasing the viscosity of the continuous phase will slow down creaming (Garti and Aserin, 1996). This can be achieved by adding a thickener (e.g. guar gum) or a gelling agent (e.g. gelatine) in the continuous phase.

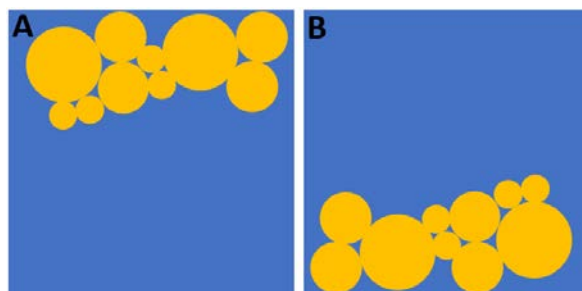


Figure 2.4 Gravitational separation of emulsion as creaming (A) and sedimentation (B).

Flocculation is the adherence of droplets to each other forming aggregates or flocculates (Figure 2.5). It occurs when the attractive forces (e.g. London-van der Waal's forces and electrostatic forces) between the droplets become greater than the repulsive forces (Branen *et al*, 2002). When flocculation occurs, it tends to increase the creaming velocity and viscosity of an emulsion (Demetriades *et al*, 1997). Flocculation can be prevented by allowing the repulsive forces to dominate the attractive forces. However, developing a suitable strategy to prevent flocculation requires the identification of the factors that causes it. For example, electrolytes added to an electrostatically stabilised emulsion screens the electrostatic repulsion between the droplets which leads to flocculation whereas sterically stabilised emulsions would not be affected (Ficheux *et al.*, 1998).

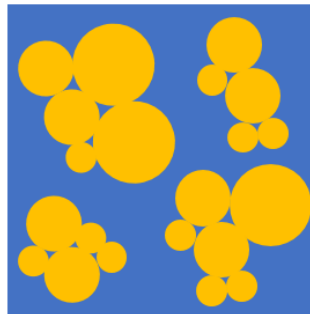


Figure 2.5 Flocculation of an emulsion with adherence of droplets to each other forming aggregates.

In emulsions coalescence is considered as the fusion of two droplets coming in close contact which is induced by hole formation in the interfacial film which propagates leading to rupture and the formation of one larger droplet (Danner and Schubert, 2001; Ficheux *et al.*, 1998; Fennema, 1996) (Figure 2.6). When two droplets collide with each other they deform and this increases the surface area between droplets in contact. The more time the droplets are in contact the higher the chances of

coalescence between them. Therefore, coalescence is more likely to occur when creaming and flocculation happens which eventually leads to phase separation. The probability that rupture of the interfacial film occurs depends on its thickness and contacting area (McClements, 2004). The thicker the interfacial film the stronger it becomes and the stronger the repulsive forces and the less likely rupture can occur (Hou and Papadopoulos, 1996). The thickness of the interfacial film can be increased by adding more emulsifier molecules or a mixture of emulsifiers (e.g. proteins and surfactants) to form multilayer films. The stability of the emulsion is also determined by the viscosity and elasticity of the W_1/O and O/W_2 interfaces (Djedour *et al.*, 2009). The visco-elastic property of interfacial film is crucial for the stability of emulsions in terms of film rupture and coalescence therefore the visco-elasticity of the film determines whether the globule will collapse or not (Myers, 1992). Increasing the concentration of surfactant leads to higher visco-elasticity and strength of the interfacial film (Vasiljević *et al.*, 2006). For example, increased adsorption of Tween80 at the interface produced more condensed membranes, able to resist hole creation and propagation that leads to film rupture (Nikiforidis and Kiosseoglou, 2011). The higher the contacting area between the droplets the more likely rupture will occur and this can be decreased by reducing the size of the droplets. Also, increasing the viscosity of the continuous phase reduces the droplets diffusion coefficient which in turn reduces droplets collision frequency and their coalescence rate. This can be achieved by adding thickeners (e.g. xanthan gum) to the continuous phase or increase the concentration of the dispersed phase (Chanamai and McClements, 2002).

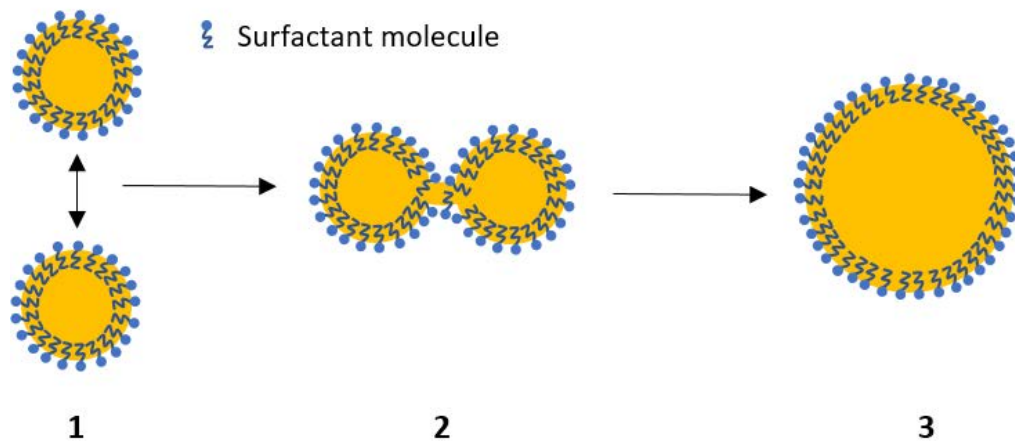


Figure 2.6 Coalescence of two droplets of an emulsion. The droplets get closer (1), hole formation occurs within the interfacial film which propagates leading to rupture (2) and formation of one larger droplet (3).

Ostwald ripening is the process by which components of the dispersed phase diffuse from smaller to larger droplets through the continuous phase (Taylor, 1995) (Figure 2.7). It occurs in emulsions with polydispersed droplets and is more often in W/O than O/W emulsions. Ostwald ripening differs from coalescence in that it's a diffusion driven mass transport where the continuous phase serves as transfer medium while in coalescence the droplets come into direct contact. For Ostwald ripening to occur the dispersed phase must be significantly soluble in the continuous phase (Dickinson, 1992). The main driving force behind Ostwald ripening is Laplace pressure and the smaller the droplets the higher the Laplace pressure and the higher the solubility compared to larger droplets (Tadros, 2013).

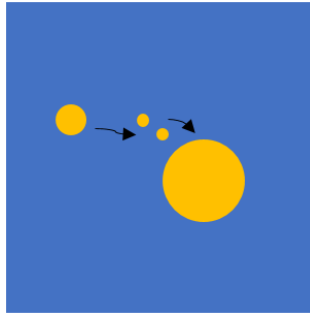


Figure 2.7 Ostwald ripening between of droplets of an emulsion. The dispersed phase component diffuse from smaller to larger droplets through the continuous phase.

Also, considered as an instability is a phenomenon known as phase inversion that occurs when phases interchange such that the dispersed phase spontaneously inverts to become the continuous phase and *vice versa* (Figure 2.8). Phase inversion is triggered by altering the emulsion composition (e.g. dispersed phase volume fraction, mechanical agitation, or surfactant concentration) (Dickinson, 1992). Emulsions that undergo phase inversion will be in a kinetically stable state. After phase inversion, an O/W emulsion changes to a W/O emulsion or vice versa.

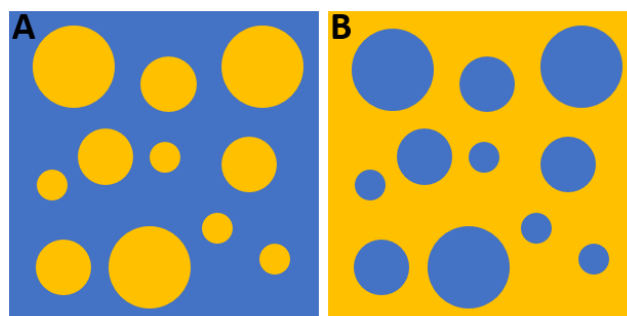


Figure 2.8 Phase inversion in an emulsion. The dispersed phase (A) becomes the continuous phase (B).

2.4.2 Instabilities in $W_1/O/W_2$ emulsions

The instabilities that occur in single emulsion also occur in double emulsions. Gravitational separation occurs in double emulsions due to differences in the density of the dispersed and continuous phases. In $W_1/O/W_2$ the dispersed phase moves upwards to form a cream layer while in $O_1/W/O_2$ emulsion the dispersed phase will move downwards to form a sediment layer. In $W_1/O/W_2$ emulsions the density of the droplets can be increased by adding electrolytes (e.g. NaCl) and hydrophilic compounds (e.g. glycerol) to the W_1 phase (Muschiolik, 2007). Other ways to reduce creaming in $W_1/O/W_2$ emulsions include reducing the size of oil globules and increasing the viscosity of the continuous phase by adding thickeners.

Flocculation in $W_1/O/W_2$ emulsion can lead to the release of the inner W_1 phase reverting to O/W emulsion (Ficheux *et al.*, 1998). In $W_1/O/W_2$ emulsions coalescence can occur between the W_1 droplets and between the W_1 droplets and O/W_2 interface. The former leads to fewer but larger W_1 droplets while the latter leads to the complete delivery of the internal W_1 droplets to the W_2 phase. The rate of coalescence between the W_1 droplets and O/W_2 interface increases when the oil droplet is destabilised. It was shown that the coalescence of W_1 droplets with O/W_2 interface increases with higher concentrations of hydrophilic surfactant. However, coalescence between W_1 and O/W_2 interface will not occur when the interfacial film thickness of the W_1 droplets is above a critical value (Ficheux *et al.*, 1998). Temperature changes can also destabilise $W_1/O/W_2$ emulsions. Thermal treatment of $W_1/O/W_2$ emulsion leads to the coalescence of W_1 droplets with O/W_2 interface (Fechner *et al.*, 2007; Iqbal *et al.*, 2013). When a $W_1/O/W_2$ emulsion is subjected to a

freeze-thaw cycle W_1 droplets coalesce with the O/W_2 interface and completely empty their contents into W_2 (Rojas *et al.*, 2008).

The osmotic pressure affects the stability of $W_1/O/W_2$ emulsions but has no effect on the stability of O/W emulsions. The movement of water between the W_1 and W_2 phases can have destabilising effects on the $W_1/O/W_2$ emulsion. When the concentration gradient is higher in the W_1 phase water will move from W_2 towards W_1 and the oil droplets will swell until the breakdown of the interfacial film occurs (Geiger *et al.*, 1998). When the concentration gradient is higher in the W_2 phase water will move from W_1 towards W_2 and the oil droplets will shrink until the $W_1/O/W_2$ emulsion becomes a simple O/W one. If the interfacial film of the globule separating the inner and outer phase is ruptured the W_1 droplets are completely delivered to the outer phase (Ficheux *et al.*, 1998). Several mechanisms have been suggested for the transport of water and water-soluble substances through the oil phase including:

- Passage through thin surfactant lamellae in the oil layer formed due to local changes of the oil layer thickness (Matsumoto *et al.*, 1976). Later it was suggested that passage of water is due to reversible thermally activated holes that are permanently formed in the bilayers (Hamilton and Kaler, 1990).
- Incorporation of water molecules within reverse micelles (Kita *et al.*, 1978; Rosano *et al.*, 1998).
- Transported via hydrated surfactant mechanism were the surfactant molecule hydrates at one interface, diffuse through the oil phase to dehydrate at the other interface that is contact with the phase of higher solute concentration (Colinart *et al.*, 1984).

Another type of transport mechanism has also been reported known as spontaneous emulsification where emulsion droplets are formed without input of energy in the form of mixing (Miller, 2006). Several mechanisms of spontaneous emulsification have been suggested including interfacial turbulence, interfacial growth due to negative interfacial tensions, and diffusion and stranding (Lopez-Montilla *et al.*, 2002). The formation of reverse micelles accelerates transport of water from W_1 to W_2 but does not disrupt the globule's interfacial film, whereas transport through thin layers of oil result in destruction of the globule's interfacial film releasing the entire inner water droplets (Garti, 1997; Lutz *et al.*, 2009a) especially during the presence of an osmotic gradient pressure between W_1 and W_2 (Florence and Whitehill, 1982). When the osmotic difference between W_1 and W_2 is very large, the flux of water becomes so fast and immediate rupture of the oil droplets is expected which leads to the delivery of the inner W_1 droplets into the continuous phase (Collings, 1971).

The rate of water transport between the W_1 and W_2 phases depends on the concentration of electrolytes in the phases (Geiger *et al.*, 1999; Wen and Papadopoulos, 2001; Cárdenas and Castro, 2003) and/or surfactants (Wen and Papadopoulos, 2000a). Increasing the concentration of NaCl in W_1 increases the swelling rate of the oil droplets leading to their breakdown (Geiger *et al.*, 1998). Wen and Papadopoulos (2000b) reported that excess Tween80 in W_2 increases the rate of water transport from W_1 to W_2 probably through mixed reverse micelles. Ficheux *et al.* (1998) showed that the lifetime of W_1 droplets within the oil globules could last from several months to few minutes depending on the concentration of Tween80. By studying single $W_1/O/W_2$ globules in a microcapillary, Wen and Papadopoulos (2000b) demonstrated that the hydrated surfactant mechanism is the dominant water

transport mechanism when the interface of W_1 droplets and W_2 are at visual contact and water transport rates increases directly with increasing NaCl concentration in W_2 . When the interface of W_1 droplets and W_2 are visually non-contacting reverse micellisation dominate and the water transport rates are independent of the NaCl concentration in W_2 . Therefore, at higher hydrophilic surfactant concentration more water is solubilised by the surfactant incorporated into the mixed reversed micelles in the oil phase, which enhances the overall mass transfer rate. However, the effects of the osmotic pressure on water transport will not be the same under different mechanisms (Wen and Papadopoulos, 2000b).

Laplace pressure is the pressure difference between the inside and the outside of a droplet, or between the convex and the concave side of a curved interface (Walstra, 1993). It works against the stability of the emulsion and stable $W_1/O/W_2$ emulsions require a balance between the Laplace and osmotic pressures. An electrolyte can be added to W_1 to counteract the Laplace pressure in droplets of $W_1/O/W_2$ emulsion; however, the amount of electrolyte must not be large enough to cause osmotic effects (Wen and Papadopoulos, 2001).

Ostwald ripening is also evident in $W_1/O/W_2$ emulsion systems (Rosano *et al.*, 1998). By adding electrolytes in W_1 of $W_1/O/W_2$ emulsion or in water droplets of W/O emulsion induces an osmotic pressure gradient in the opposite direction which will counteract the Laplace pressure effect (Wen and Papadopoulos, 2001). Also by ensuring that the droplet size distribution is kept narrow Ostwald ripening can be retarded (Kabalnov and Shchukin, 1992). Phase inversion also occurs in $W_1/O/W_2$

emulsions (Allouche *et al.*, 2004). For example, phase inversion will occur at high water content and a $W_1/O/W_2$ emulsion changes to a simple O/W emulsion.

2.5 Methods of emulsification

The methods that are used for emulsification are categorised as high-energy, intermediate-energy and low-energy, and require the use of devices that deliver the energy needed to form the emulsions (McClements, 2004). The emulsification production procedures as well as the disadvantages of each method will be discussed in the section below.

2.5.1 High-energy emulsification methods

High-energy emulsification methods are used by industry for forming emulsions at large scale using conventional emulsification devices such as rotor-stator, high-pressure homogenisers, and ultrasonic transducers. These devices provide high shearing rates and energy inputs per unit volume (McClements, 2004).

Rotor-stators also known as high shear homogenisers (e.g. Silverson) are widely used by industry to manufacture emulsion-based foods, cosmetics, pharmaceuticals and health care products (Hall *et al.*, 2011; Zhang *et al.*, 2012). All rotor-stator devices consist of a rotating shaft (rotor) inside a stationary tube (stator) and the tip of the stator is designed to have slots or a perforated screen while the rotor serves as a blade. In rotor–stators liquid disruption occurs under turbulent flow conditions to form droplets and these can be further ruptured by two main mechanisms, one due to the rotor and one due to the stator (Roger and Cooke, 2012). The high-speed rotation of the rotor (ranging from 10 to 50 m/s) will create a powerful suction drawing

the liquids upwards into the working head (Zhang *et al.*, 2012). Centrifugal force then drives the liquids towards the periphery of the work head where they are rapidly milled between the rotor blade and the stator. This is followed by an intense hydraulic shear as the liquids are forced out through the slots and back into the mix (Hall *et al.*, 2011) (Figure 2.9). Rotor-stators consume high amounts of energy (up to 10^7 Jm^{-3}) due to the centrifugal forces generated from the movement of the rotor and the stator equipped with narrow spacing (Walstra and Smulders, 1998). The emulsions that are formed by rotor-stators have a wide droplet size distribution which may not be desirable in certain applications. Emulsification using rotor-stators is not suitable for forming emulsions that contain sensitive ingredients that can be disintegrated with high shear stress (e.g. proteins) (McClements, 2004). Also, they are limited in terms of the droplet size (diameter $>0.5\mu\text{m}$) that can be achieved and after mixing an additional emulsification method may be required (e.g. ultrasound sonication or high-pressure homogenisation) to further reduce the droplet size (Zhang *et al.*, 2012). Parameters that affect droplet break-up during rotor stator homogenisation includes speed and duration of mixing as well as the temperature of the liquids during mixing and device parameters such as number and size of perforations of screen on the stator (McClements, 2004).

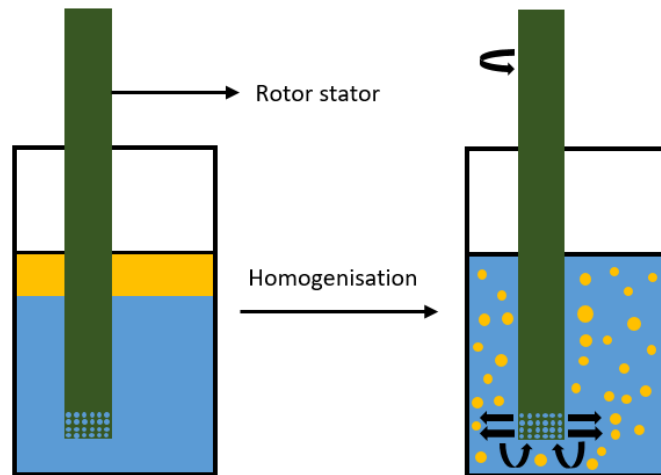


Figure 2.9 High-shear homogenisation. High-speed rotation of the rotor creates a suction drawing the liquids upwards into the working head and centrifugal forces drives the liquids towards the periphery of the work head where they are rapidly milled between the rotor blade and the stator.

High-pressure homogenisers are also used by industry to manufacture emulsion-based foods, cosmetics, pharmaceuticals and health care products (Zhang *et al.*, 2012). High-pressure homogenisers also exert turbulence fragmenting forces using high shear in order to break-up the droplets until they the desired droplet size is achieved (Håkansson *et al.*, 2012). All high-pressure homogenisers have two major components: a high-pressure pump and a homogenising nozzle or valve (Figure 2.10). The coarse emulsion is forced into a narrow gap using high-pressure (100-2000 bar) produced by the pump that compresses the emulsion. During depressurisation in the homogenising nozzle or valve the droplets are ruptured by turbulence and cavitation to very small size (up to $0.1\mu\text{m}$ in diameter) (Gijsbertsen-Abrahamse *et al.*, 2004). Before high-pressure homogenisation the liquids need to be pre-emulsified using a rotor-stator to form a coarse emulsion that can be further homogenised into a fine emulsion. High-pressure homogenisation requires high

amounts of energy (up to 10^8 Jm^{-3}) (Walstra and Smulders, 1998). The emulsions that are formed by high-pressure homogenisers have a wide droplet size distribution which may not be desirable in certain applications. It is also not suitable to form emulsions that contain sensitive ingredients that can be disintegrated with high shear. Also, the high-pressure causes an increase in temperature which can damage certain heat sensitive components of the emulsion (e.g. denature proteins). It is also difficult to sterilise and/or clean which makes it limited in terms of certain applications. Parameters that affect droplet break-up during high-pressure homogenisation include the flow rate, pressure, number of cycles, temperature, and device parameters such as nozzle or valve geometry and design (McClements, 2004).

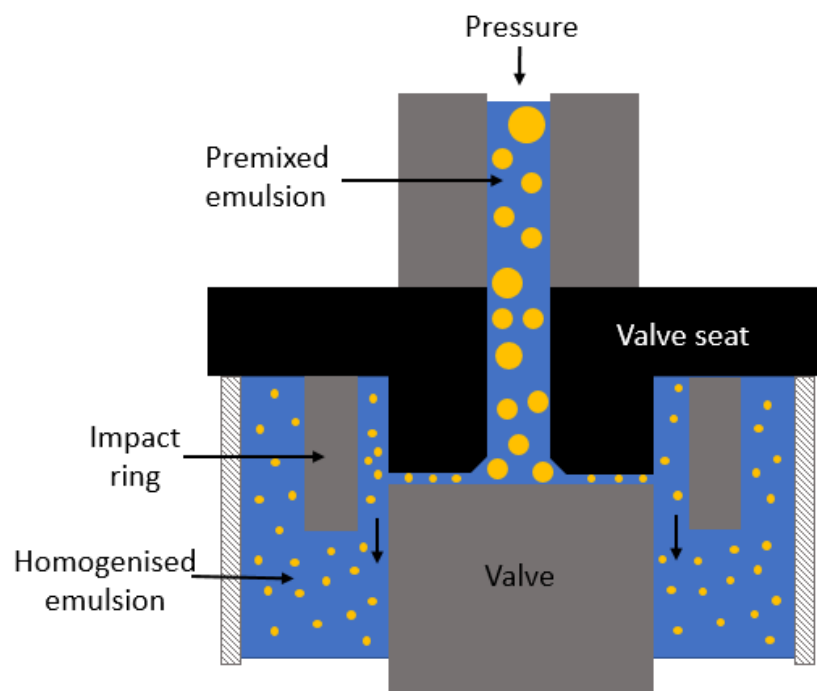


Figure 2.10 High-pressure homogenisation. The coarse emulsion is forced into a narrow gap using high-pressure where the droplets are ruptured by turbulence and cavitation.

Ultrasound homogenisation is mainly used to form small quantities of emulsion therefore it is more frequently used at lab scale. Ultrasound in the range 16-100kHz can induce physical changes to liquids by cavitation phenomena while sonication greater than 20kHz is required to form emulsions with nano-sized droplets (Leong *et al.*, 2009). Ultrasound emulsification can achieve droplets size of down to 0.1µm in diameter (Jafari *et al.*, 2006). There are two techniques of sonication; direct and indirect. In the latter, the ultrasonic waves are transmitted via a water bath through the container's wall and into the liquid. Since the ultrasonic waves must travel through water bath and container the ultrasonic intensity is low therefore it is not commonly used for homogenisation. In direct sonication, a probe-type sonicator is used whereby an ultrasonic horn or sonotrode is immersed into the liquid and ultrasonic waves are directly transmitted via the sonotrode at high intensity (Gaikwad and Pandit, 2008). Ultrasound causes the formation and collapse of vapour cavities leading to extreme levels of highly localised turbulence and impact pressure which ruptures the droplets (Jafari *et al.*, 2006) (Figure 2.11). Compared to other homogenisation devices probe-type sonicators have lower number of wetted parts which reduces the frictional wear and cleaning time. Due to its simple geometry and absence of hidden orifices the sonotrode can be sterilised if required and therefore can be used aseptically to prevent contamination. Compared to high-pressure homogenisation, ultrasonic homogenisation is more energy efficient as less energy is needed to make droplets of the same size (McClements, 2004). Ultrasound homogenisation consumes high amounts of energy (up to 10^7 Jm^{-3}). It also causes an increase in temperature which can damage certain heat sensitive components of the emulsion (e.g. denature proteins). In ultrasound emulsification, it is possible to

directly form an emulsion from the separate phases but this would need a great amount of energy and therefore it is better to do a pre-emulsification step using a rotor-stator to form a coarse emulsion before applying acoustic power (Canselier *et al.*, 2002). The emulsions that are formed by ultrasound transducers have a wide droplet size distribution which may not be desirable in certain applications. Parameters that affect droplet break-up during high ultrasonic homogenisation include the power, frequency and duration of sonication and device parameters such as the diameter of the sonotrode (McClements, 2004).

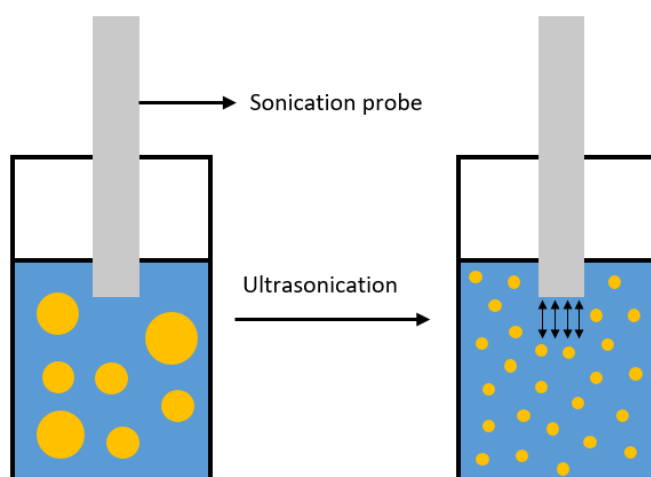


Figure 2.11 Ultrasonic homogenisation. Ultrasound waves are directly transmitted via the sonotrode at high intensity generating interfacial waves to form droplets by dispersing the oil phase into the continuous phase.

2.5.2 Intermediate-energy emulsification methods

Intermediate-energy emulsification methods are used on lab scale and not commonly used for large scale industrial applications. The most common devices used include the membrane homogenisers and microfluidic systems. These devices provide intermediate shearing rates and energy inputs per unit volume (McClements, 2004).

Membrane homogenisers are used to form emulsions by forcing the dispersed phase into a porous membrane (e.g. glass) and at its surface droplets will form, detach and get carried away by the continuous phase flowing across the surface of the membrane (Charcosset *et al.*, 2004) (Figure 2.12). Membrane homogenisation can either be batch or a continuous process. The batch or stirred cell process droplets are formed when the dispersed phase is forced through a cylindrical membrane immersed in a vessel containing the continuous phase and stirred using a mixer. The continuous or cross-flow process, the continuous phase flows through a cylindrical membrane which is located within a tube where the dispersed phase is flowing. The dispersed phase is forced to pass through the membrane to form droplets in the continuous phase (Deluca *et al.*, 2007; Timgren *et al.*, 2010). Both processes are highly energy efficient and can produce emulsions with a narrow droplet size distribution and can achieve droplet size of down to 0.3µm in diameter (McClements, 2004).

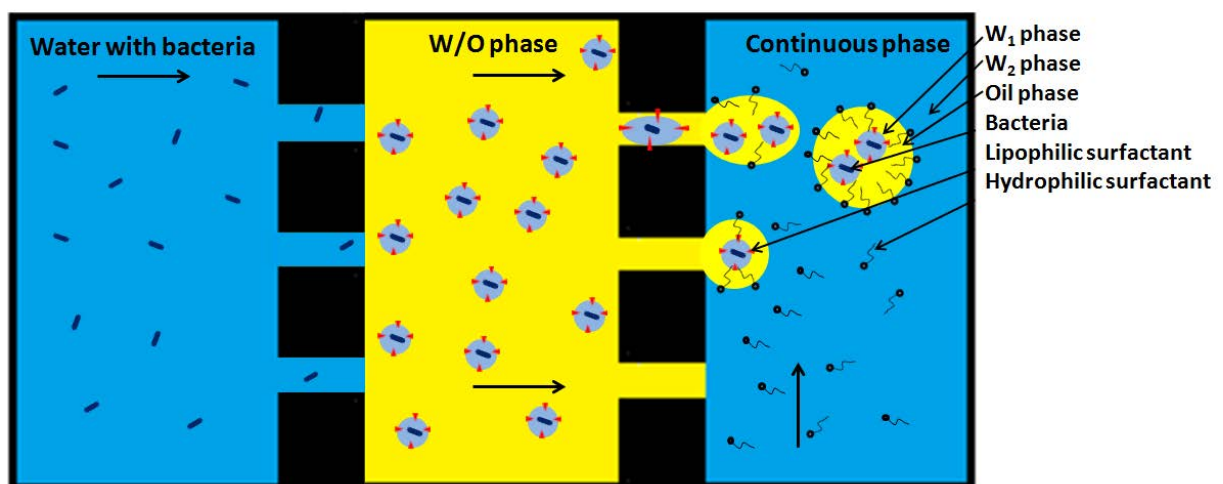


Figure 2.12 Membrane homogenisation. Dispersed phase is forced into a porous membrane and at its surface droplets will form, detach, and get carried away by the continuous phase flowing across the surface of the membrane. The direction of the flow of the phases is shown by a black arrow.

Microfluidic emulsification is currently used on lab scale and industrial application is not yet possible due to low throughput. In microfluidic emulsification, a pump (e.g. syringe pump) is used to produce a pressure driven laminar flow of the dispersed and continuous phases through channels and where they meet a junction, droplets can be formed (Shah *et al*, 2008) (Figure 2.13). This method can produce emulsions with very narrow droplet size distribution and can form double emulsions where the size and number of inner droplets are manipulated to unprecedented accuracy (Maan *et al.*, 2015). Parameters that affect droplet detachment during microfluidic emulsification include the pressure, flow rate, temperature and device parameters such as the channel size (Shah *et al*, 2008).

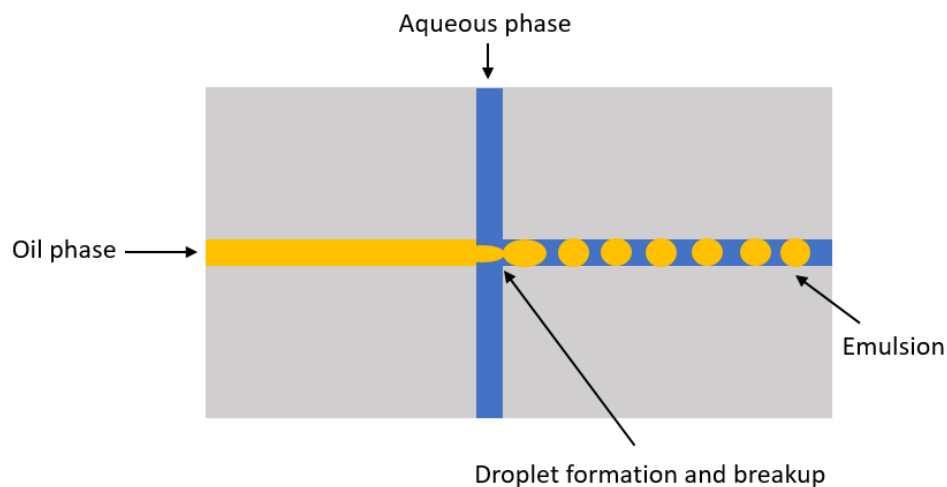


Figure 2.13 Microfluidic emulsification. The dispersed phase is driven by pressure into the continuous phases through channels and at a junction formation and break-up of droplets occurs.

2.5.3 Low-energy emulsification methods

Low-energy emulsification methods utilise the stored energy of the system to form emulsions. Although low-energy emulsification methods have applications in industry

they are not as common as high-energy methods. Low-energy emulsification methods include spontaneous emulsification (SE), catastrophic phase inversion (CPI), transitional phase inversion (TPI), phase inversion temperature (PIT) and emulsion inversion point (EIP) methods. In SE, the formation of emulsion takes place spontaneously by mixing an organic phase with an aqueous phase at a particular temperature. Under certain conditions, pouring a mixture of a totally water miscible solvent and a hydrophobic oil into water, generates spontaneously small droplets which are stable in the absence of an emulsifier (Solans *et al.*, 2016). A good example of this is a phenomenon known as the Ouzo effect which occurs when an anise flavoured beverage such as Arak that contain Anatole oil and ethanol is dispersed in water. Since the essential oil of anise is soluble in alcohol but not in water the Arak spontaneously nucleates into many small droplets without the help of emulsifiers or shear rate of any kind (Vitale and Katz, 2003). Phase inversion is a phenomenon that occurs when the dispersed phase in an emulsion becomes the continuous phase and *vice versa*. In CPI, the continuous phase volume fraction is reduced by evaporation which causes the dispersed phase volume fraction to exceed a certain value that leads to an irreversible phase inversion (Salager *et al.*, 2000). In TPI the emulsifier's affinity changes towards the dispersed phase caused by changes in temperature, salt concentration or pH leading to a reversible phase inversion (Antón *et al.*, 1986). In PIT, the emulsifier's HLB is altered by the changing the temperature. The liquid phases are added with the emulsifier at their phase inversion temperature also known as HLB temperature which is the temperature where the solubility of the emulsifier in either phase is equal (Lehnert *et al.*, 1994). The rapid heating or cooling of the solutions will form a W/O or O/W emulsion respectively

(Morales *et al.*, 2003). In EIP the change in composition of the system through diluting a mixture of oil and emulsifier by adding water at a constant temperature leads to a change in the spontaneous curvature of the emulsifier and thus changing its affinity and the emulsion will change from W/O to O/W emulsion (Fernandez *et al.*, 2004). These methods are highly energy efficient and can achieve emulsions with droplets of less than 0.1 μm in diameter and narrow size distribution.

2.6 Characterisation of emulsions

Characterisation techniques include size distribution, ζ -potential and rheology. Different techniques have been developed to measure droplet size distribution; the most common techniques are microscopy (Davis, 2005) and light scattering (Richardson, 2005; Kiokias *et al.*, 2004; Lindner *et al.*, 2001).

2.6.1 Light scattering techniques

Light scattering techniques involve shining a laser beam through the emulsion to be measured and the intensity distribution caused by the scattering of the droplets is picked up with a detector (Figure 2.14). Static and dynamic light scattering are the most common used light scattering techniques for measuring droplet size in emulsions (McClements, 2004). Static light scattering (SLS) measures droplet size based on the detection of diffracted light (Dickinson and Stainsby, 1982). A monochromatic light beam generated by a laser strikes the border of a droplet which gets illuminated generating a specific interference pattern known as the Fraunhofer diffraction rings. In addition to the diffraction phenomena, the intensity of scattered light is the sum of reflection, adsorption, refraction and reradiation. The intensity of the scattered light at one or many

angles is measured by detectors and the droplet size is calculated using the Mie theory which describes all effects from incident light on a spherical particle. The Mie theory requires some information on optical properties (e.g. refractive index) of the particle being measured and the dispersant (Zhang and Xu, 1992). Dynamic light scattering (DLS) measures the velocity of light scattered from the dispersed droplets in Brownian motion (Hunter, 1986) and converts the intensity of scattered light to droplet size using the Stokes-Einstein equation assuming droplets are spherical in shape (Horne, 1995). There are two instruments that can measure droplet size; Mastersizer that uses SLS and Zetasizer that uses DLS. Mastersizer can measure droplets in the range of 0.1-2000 μm while the Zetasizer can measure droplets starting from 1nm to a few microns in size (Malvern, 2016).

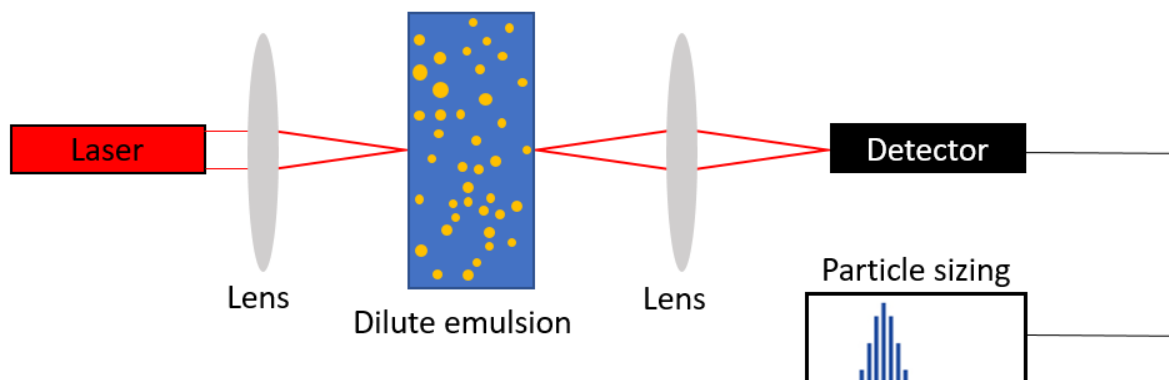


Figure 2.14 Light scattering technique. The laser beam is directed towards the emulsion striking the droplets and the intensity of the scattered light is recorded using a detector and a data acquisition system.

2.6.2 ζ -potential (zeta potential)

In emulsion systems, the continuous phase may contain ions and some will be close to the surface of the droplets and are strongly bound forming the stern layer while the further ones are loosely bound forming the diffuse layer (McClements, 2004). Ions within the diffuse layer will move with the droplets as they move within the continuous phase and these are within a notional boundary called the Slipping plane and ions outside this boundary will remain where they are. A potential exists between the surface of the droplet and the continuous phase which varies according to the distance from the surface of the droplet and this potential at the Slipping plane is called the ζ -potential (Figure 2.15). The Zetasizer can measure the ζ -potential of the droplets in an emulsion (Hunter, 1986). After the emulsion is placed in a folded capillary cell that has two electrodes an electric field of known strength is applied and droplets will drift towards the electrode that has opposite charge to theirs. The software of the Zetasizer will measure the drift velocity and calculate the ζ -potential by application of the Henry equation (Hunter, 1988). Bacterial cells can be present in emulsions due to contamination or as part of the microflora. The stability of the emulsion depends on the ζ -potential of the droplets and bacterial cells present in the aqueous phase. It has been reported that when ζ -potential of the emulsion and the bacteria were opposite to each other the emulsion becomes unstable as coalescence and flocculation increased (Ly *et al.*, 2006; Ly *et al.*, 2008).

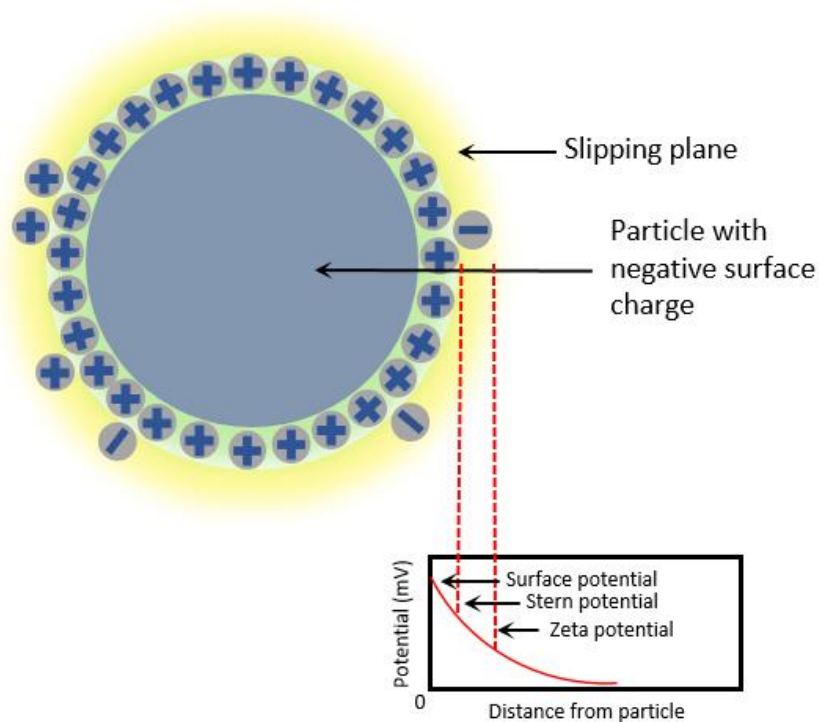


Figure 2.15 Zeta potential. Diagram showing the ionic concentration and potential difference as a function of distance from the charged surface of a particle suspended in a dispersion medium.

2.6.3 Rheology

The rheological behaviour (e.g. viscosity) of an emulsion is an indicator of instability. Viscosity is a measure of the resistance of emulsion to an applied stress and emulsion viscosity depends on the initial viscosity of the continuous phase and the dispersed phase volume fraction (Pal, 1996). The viscosity of an emulsion can be studied by different instruments which can be categorised according to whether they are based on simple compression (or elongation) or shear forces (Macosko, 1994). Simple compression and elongation is carried on solid or semisolid emulsions using instruments referred to as Universal Testing Machines. It consists of a probe that exerts lateral force on the sample that is placed on a plate. The probe and the plate

contain a pressure sensor to measure the force exerted on the sample. By recording the distance, the probe travelled through the sample (deformation) the instrument can calculate the stress and strain experienced by the sample (Whorlow 1992; Macosko, 1994). Shear measurements are carried on liquid and solid emulsions using instruments referred to as rheometers. There are two most common types of rheometer used to measure the viscosity of an emulsion including constant stress instrument where a constant torque is applied on the sample and the resultant strain is measured or constant strain instrument which applies strain and measure the torque generated in the sample.

Visco-elasticity is the property of materials that exhibit both viscous and elastic behaviour simultaneously when undergoing deformation. Emulsions have rheological properties that are partly viscous and partly elastic (McClements, 2005). A visco-elastic material does not instantaneously take-up its new dimensions when a force is applied and when the force is removed the material does not instantaneously return to its non-deformed state (as an ideal elastic material would), and it may even remain permanently deformed. Furthermore, visco-elastic interfacial films promote emulsion stability in emulsions. There are two types of experimental tests used to characterize the visco-elastic properties of emulsions: transient and dynamic measurements (Whorlow 1992). Both types of tests can be carried out by the application of simple shear, simple compression, or bulk compression to the material being analyzed using a rheometer. However, simple shear tests are the most commonly used for analyzing emulsions. In a transient experiment a constant force is applied to a material and the resulting strain is measured as a function of time, or *vice versa*. Transient experiments includes creep and stress relaxation experiments. In a creep experiment

a constant stress is applied and whereas in a stress relaxation experiment a constant strain is applied and the corresponding stress is followed as a function of time. In a dynamic experiment a sinusoidal stress is applied to a material and the resulting sinusoidal strain is measured, or *vice versa* (Whorlow 1992).

2.7 Microscopy

Several microscopic techniques have been used to observe the structure of an emulsion and this includes optical, fluorescence, scanning electron microscopy and each works on different physicochemical principles.

The most common optical microscopy techniques used to observe emulsions are the bright-field and phase-contrast microscopy (Mikula 1992; Hunter, 1993). In bright field microscopy, visible light from a light source (e.g. bulb) will pass through a condenser lens (focuses light) and then through the sample that is placed on a glass slide. The light that is then refracted around, or reflected from the sample then passes through the objective lens and then the eyepiece lens before it reaches the eye. Phase-contrast microscopy is like bright-field microscopy but is able to give higher contrast by exploiting differences in the refractive index of different structures being observed. A phase-contrast microscope uses an annular stop within the condenser that is aligned with a phase plate within the objective lens to bend light and delay its transmission through the sample at varying degrees. Fluorescence microscopy has been commonly used to observe emulsions (Ly *et al.*, 2006; Ly *et al.*, 2008; Miesch *et al.*, 2012; Sugumar *et al.*, 2013). A fluorescent microscope is an optical microscope that uses fluorescence to excite a fluorescent dye that is bound to specific parts of the sample. The light (e.g. argon laser) with the appropriate

wavelength will pass through a filter that only allow light with a certain wavelength to pass and illuminate the sample. In the fluorescent dye, the electrons in the fluorophores (fluorescent molecules) get to an excited state and then fall down to a ground state losing energy in the form of emitted light. The light emitted has lower energy and a longer wavelength than the excitation light and this is known as the Stoke's-shift (Figure 2.16). The fluorescent microscope has a dichroic mirror and a second filter which only allow the emitted light to pass before reaching the eyepiece (Sanderson *et al.*, 2014). All optical microscopes are limited in terms of their magnifications and resolution (McClements, 2004).

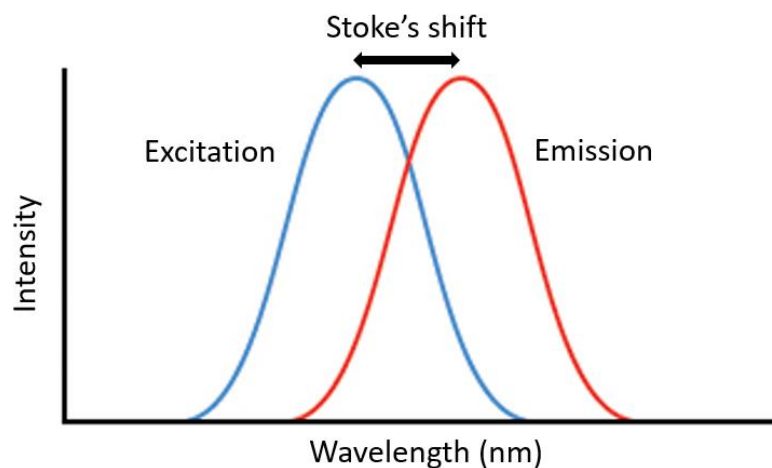


Figure 2.16 Stoke's-shift. The loss in energy after excitation leads to emitting at a longer wavelength.

Electron microscopes give higher magnification and resolution compared to optical microscopes (Hunter 1993). In SEM, a 3D-image is produced by the electrons bouncing off the sample and detected by multiple detectors. An electron gun is used to generate the beam of electrons which is focused using condensers (Figure 2.17). Cryofracture-SEM (cryo-SEM) has been utilised to observe emulsions (Frasch-Melnik *et al.*, 2010a; Frasc-Melnik *et al.*, 2010b; Jaimes-Lizcano *et al.*, 2013) and it works

in a similar manner as SEM but the sample needs to be frozen very rapidly using liquid nitrogen and then fractured.

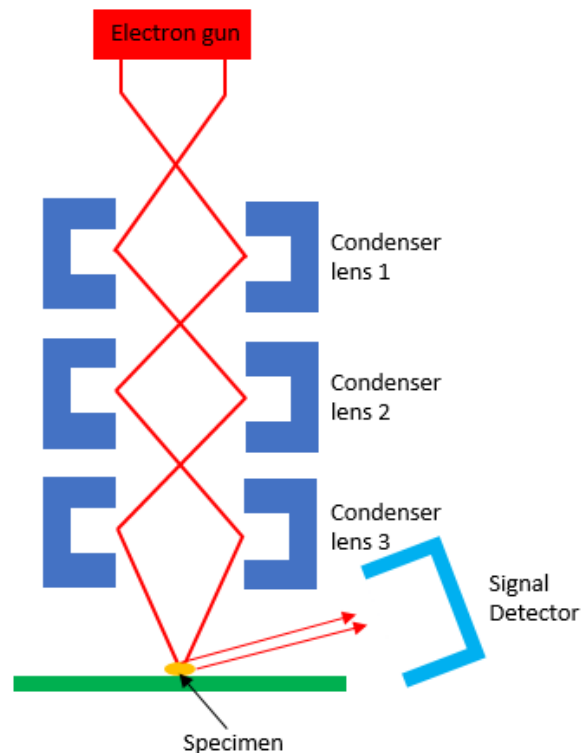


Figure 2.17 Scanning electron microscope. The electron gun generates the beam of electrons which is focused using condensers. The electrons are reflected from the specimen and collected by the detector.

2.8 Effect of bacteria on stability of emulsions

The presence of bacteria can affect the stability of emulsions. Depending on bacterial physicochemical surface properties and metabolic activity the stability of the emulsion can be altered. The properties of bacteria that lead to altering emulsion stability will be discussed below.

2.8.1 Physicochemical changes in emulsions caused by bacteria

When microorganisms are present in food-based emulsions as part of the microflora or due to contamination they can grow and result in spoilage (Brocklehurst and

Wilson, 2000). Food-based O/W emulsions have a nutrient content that is suitable for utilisation by spoilage bacteria. Yeasts such as *Zygosaccharomyces bailii* and lactic acid bacteria such as *Lactobacillus fructivorans* and *Lactobacillus brevis* are common spoilers in mayonnaise and salad dressings (Muriana and Kanach, 1995; Kurtzman *et al.*, 1971). *Bacillus vulgatus* has been reported to spoil mayonnaise-based salad dressing (Pederson, 1930). In W/O emulsions spoilage is less common than in O/W emulsion because the microorganisms in water droplets are compartmentalised which limits their spread throughout the emulsion (Brocklehurst and Wilson, 2000).

Spoilage usually leads to the destabilisation of the O/W emulsion indicated by a rapid phase separation and breakdown of the emulsion structure (O'May *et al.*, 2004). Bacterial growth can be visible in emulsions during spoilage and they undergo chemical (e.g. drop in pH) and physical changes (e.g. changes in viscosity) (Gillatt, 1991). The darkening of the emulsion due to microbial growth along with gassing and organoleptic rancidity are the first signs of spoilage followed by the separation of the dispersed and continuous phases (Erkmen and Bozoglu, 2016). Bacteria will utilise the nutrients (e.g. sugars) present in the aqueous phase and alter the pH usually through acid production (O'May *et al.*, 2004). This can affect the solubility of the surfactants and in turn accelerate the process of emulsion breakdown. The bacteria can consume certain components of the emulsion that are essential for stability (e. g. a surfactants or thickening agents). Some bacterial species have shown to utilise Tween80 as a carbon source. When cultivated in medium containing Tween80 the bacterium *Microthrix parvicella* utilised the oleic acid moiety of Tween80 as a carbon and energy source (Slijkhuis *et al.*, 1984). Strains of *Pseudomonas fluorescens* and *P. aeruginosa* are able to grow on minimal medium in the presence

of Tween80 as a carbon source (Howe and Ward, 1976). Several species of Gram-negative and Gram-positive bacteria have shown to ferment various polymers (e.g. starch and guar gum). In paint industry spoiler bacteria produce cellulase enzyme which is capable of breaking down long chain cellulosic thickening agents resulting in loss of viscosity (Gillatt, 1991).

2.8.2 Extracellular molecules and surface properties of bacteria and emulsion stability

Bacteria have the ability to modify emulsion properties due to their hydrophobic cell surfaces or the dual amphiphilic nature of their extracellular molecules (e.g. biosurfactants) which displace or alter emulsifiers adsorbed at the interface and destabilise the emulsion (Das, 2001). Biosurfactants are amphiphilic molecules containing a hydrophilic group (head part) and a hydrophobic group (tail part) and are either attached to the surface of the cell membrane or excreted extracellularly (Desai and Banat, 1997). Biosurfactants can either stabilise (Sousa *et al.*, 2014, Pekdemir *et al.*, 2005) or de-stabilise (Huang *et al.*, 2013; Long *et al.*, 2013; Coutinho *et al.*, 2013) an emulsion. A variety of bacteria can produce biosurfactants; *P. aeruginosa* and *P. putida* excrete glycolipids (e.g., rhamnolipids) and *Bacillus subtilis* excrete lipopeptides (e.g. surfactin) (O'May *et al.*, 2004).

Emulsion stability also depends on the surface properties of the bacteria and characteristics of the emulsions. Li *et al.* (2001) studied the effect of *E. coli* JM109 and *E. coli* E21 strains that possess different surface charge (-35mV and -5mV respectively) on the stability of O/W emulsions made with Tween20 (non-ionic), sodium dodecyl sulfate (SDS) (anionic) or dodecyltrimethylammonium bromide

(DTAB) (cationic). Both strains had no effect on the stability of the emulsions made with Tween80 and SDS, however, flocculation and coalescence along with phase separation occurred in emulsions made with DTAB but at a much faster rate with *E. coli* JM109 compared to *E. coli* E21. In another study Ly *et al.* (2006) tested the effect of different strains of *Lactococcus lactis* subsp *lactis* biovar diacetylactis with different surface charge and hydrophobicity on the stability of O/W emulsions made with Tween80 (non-ionic), SDS or cetyltrimethylammonium bromide (CTAB) (cationic). When the bacterial strains possessed an opposite charge to that of the surfactant, flocculation and creaming occurred. Using fluorescence microscopy, they observed that the droplets became larger in size and the cells were adsorbed at the surface of the droplets forming bridges between them leading to flocculation and coalescence. In a later study, they tested the effect of the same *L. lactis* strains on the stability of O/W emulsions made with whey protein isolate (WPI), whey protein concentrate (WPC) or sodium caseinate (Ly *et al.*, 2008). Their previous observation was confirmed in that when the bacterial strains possessed an opposite charge to that of the surfactant, flocculation and creaming occurred.

On the other hand microorganisms can stabilise the emulsion by behaving as fine solid particles at interfaces. In thermally-inactivated form, bacteria and yeast cells can be used as micron-sized Pickering colloidal particles. Different bacterial species were able to stabilise O/W and W/O emulsions including *Acinetobacter venetianus* RAG-1 and *Rhodococcus erythropolis* 20S-E1-c (Dorbantu *et al.*, 2004). Confocal microscopy images showed that bacterial cells were surrounding the oil droplets. Recently, thermally-inactivated baker's yeast (*Saccharomyces cerevisiae*) (Moreira *et al.*, 2016) and lactic acid bacteria (*Lactobacillus acidophilus* and *Streptococcus*

thermophilus) were used to generate and stabilise O/W emulsions (Firoozmand and Rousseau, 2016). A biobased material based on a bacteria–chitosan network (BCN) was developed using the electrostatic interaction between negatively charged bacterial cells and polycationic chitosan. The BCN was proven to stabilize the interface promoting formation of highly stable O/W emulsion (Wongkongkatep *et al.*, 2012).

2.8.3 Metabolic activity of bacteria and emulsion stability

Bacteria can also alter the osmotic balance of the aqueous phase by excreting waste that could alter the stability of the emulsion. Boitard *et al.* (2012) found that when *E. coli* cells were encapsulated in water droplets of W/O emulsion containing glucose as carbon source they excrete waste products leading to osmotic mismatch that causes droplet shrinkage. The authors show that the shrinking rates directly and quantitatively reflect glucose consumption and that droplets with high bioactivity show progressive decrease in size at rates depending on the initial concentration of glucose, strain, species, and number of the bacteria cells (Boitard *et al.*, 2012).

2.9 Effect of emulsion structure on bacterial survival, growth, and stress response

The structure of emulsions can affect bacteria in the aqueous phase. Altering emulsion structure (e.g. reducing droplet size) can affect the growth rate of bacteria and determine whether the bacteria grow as planktonic (as single cells) or colonies. Properties of emulsions that can affect bacteria are discussed below.

2.9.1 Space limitation in emulsion and bacterial growth

The structure of the emulsion can affect the growth of bacteria. Studies have shown that Gram-negative and Gram-positive bacteria are constrained to grow as colonies instead of planktonic cells when the volume of the oil phase was increased. Parker *et al.* (1995) tested the form of growth of *Listeria monocytogenes*, *Salmonella typhimurium* and *Yersinia enterocolitica* in O/W emulsions made with 83% hexadecane as the dispersed phase. Using SEM, they found in aqueous phase of equivalent chemical composition bacteria grew as planktonic cells whereas they were restricted to grow as colonies in the emulsions. It was found that the microstructure of emulsions containing 83% oil immobilised the bacteria which were constrained to grow as colonies. In a similar study, the same group tested the form of growth of *L. monocytogenes* and *Y. enterocolitica* in O/W emulsions made with 30, 70 and 83% hexadecane or sunflower oil as the dispersed phase. Using SEM, they found that bacteria grew as planktonic cells in emulsions containing 30 and 70% oil whereas they were restricted to grow as colonies in emulsions containing 83% oil. The authors concluded that in emulsions containing 83% oil bacteria formed colonies due to restricted diffusion of nutrients and oxygen or accumulation of waste products of metabolism (Brocklehurst *et al.*, 1995).

Prachaiyo and McLandsborough (2003) studied the formation of curli (surface structures involved in bacterial attachment to surfaces) and ability to grow in stressful environments (heat stress) in pathogenic *E. coli* 0157:H7 cells grown in O/W emulsion containing 20% or 40% hexadecane as the dispersed phase as opposed to liquid media. Curli formation is induced under stressful environments and the increase in curli expression in *E. coli* is usually an indicator of stress (e.g. starvation).

Using SEM, they found that bacteria formed curli when grown in emulsion as opposed to just liquid media. They also quantified curli formation by plating on Congo red indicator plates (CRI), a method for quantification of curli formation and found that curli expression was higher when the concentration of the oil phase was increased from 20 to 40%. With increasing oil phase concentration, the bacteria were found to become more heat resistant.

Boedicker *et al.* (2009) investigated quorum sensing (QS), a process of cell-cell communication, in *Pseudomonas aureginosa* that express the green fluorescent protein (GFP) as a QS reporter. The process of QS is necessary for growth, biofilm formation, motility, and other critical functions in bacteria. They showed that when confined in a small volume *P. aureginosa* initiate QS that was observed microscopically as an increase in GFP expression inside water droplets.

2.9.2 Droplet size in emulsion and bacterial growth

Reducing the droplet size can impact the growth of bacteria in emulsions. Brocklehurst *et al.* (1995) investigated the effect of changing the droplet size (mean diameter of 2µm, 15µm or 25µm) in O/W emulsions containing hexadecane or sunflower oil (dispersed phase) on the form of growth of *L. monocytogenes* and *Y. enterocolitica*. The viscosity of the emulsions increased with decreasing droplet size. Using SEM, they found that bacteria formed small colonies in emulsions with mean droplet size of 2µm while in emulsions with mean droplet size of 15µm they formed small colonies and single-cell-thick films. However, bacteria formed single-cell-thick films or grew as planktonic cells in emulsions with mean droplet size of 25µm. They also found that the growth rate of bacteria was inhibited in emulsions with mean

droplet size of 2µm but not in emulsions with mean droplet size of 15µm-25µm. The authors concluded that emulsions with mean droplet size of 2µm the droplets were sufficiently close-packed and viscous which immobilised the bacteria. On the other hand, the emulsions with larger mean droplet size (15µm and 25µm) were less viscous which did not prevent the mobility and therefore growth rate of bacteria was not inhibited (Brocklehurst *et al.*, 1995).

The physical properties of nano-emulsions can be quite different from micro-emulsions and therefore may influence growth and survival of microorganisms. The nano-sized droplets in nano-emulsions possess high surface tension which can disrupt bacterial cell membrane. Several studies investigated the effect of nano-emulsions on the survival of bacteria. Karthikeyan *et al.* (2011) investigated the effect of a nano-emulsion formulation with droplets of mean diameter size of 308nm compared to its separate components on the survival of *Streptococcus mutans* in planktonic form and biofilm. A serial dilution technique was used for the determination of minimum inhibitory concentration and minimum bactericidal concentration (MIC/MBC). The nano-emulsion showed higher antibacterial activity for both planktonic and biofilm bacteria. Using SEM, an extensive disintegration of the cell membrane, disruption to cell wall and lysis of *S. mutans* was evident after treatment with nano-emulsion (Karthikeyan *et al.*, 2011). In a later study, the same group conducted a study to investigate the effect of a nano-emulsion formulation with droplets of mean diameter size of 308nm compared to its separate components on the survival of various strains of bacteria in planktonic form and biofilm (Karthikeyan *et al.*, 2012). The nano-emulsion showed higher antimicrobial activity (determined by MIC/MIB) against biofilms formed by *S. mutans* and *Lactobacillus casei* as 83% loss

in viability within 1 hour. Using fluorescence microscopy staining with the Live/Dead BacLight bacterial viability kit they found a higher number of dead cells after exposure to nano-emulsion. The level of bacterial adhesion to glass (essential for biofilm formation) was reduced by 94.2-99.5% in the nano-emulsion treated group. Nano-emulsion showed higher antimicrobial activity against planktonic cells of *S. mutans*, *L. casei*, *Actinomyces viscosus* and *Candida albicans* and a mixed culture of the four strains (Karthikeyan *et al.*, 2012). In another study, it was shown that after treatment with nano-emulsion with droplets of mean diameter size of 17.1nm the membrane integrity of *Bacillus cereus*, *Staphylococcus aureus* and *E. coli* was compromised which was evident by leakage of the cytoplasmic content measured using UV absorbance (Sugumar *et al.*, 2013). In another study *E. coli* cells treated with nano-emulsions with droplets of mean diameter size of 29.6nm showed deformation in bacterial membrane phospholipids confirmed by FT-IR analysis. Also, using fluorescence microscopy these cells stained positive with ethidium bromide, a stain that stains the DNA of cells with a membrane that lost its structural integrity (Ghosh *et al.*, 2013).

The antimicrobial effect of nano-emulsions has been attributed to their structure itself and the nano-sized droplets. When nano-emulsions are formed under high shearing forces (e.g. ultrasonication, high-pressure homogenisation or high-shear mixing) they acquire significant amount of energy as they are formed (Lee *et al.*, 2010). The nano-droplets are thermodynamically driven to fuse with lipid-containing micro-organisms and the energy that was stored during formation of the nano-emulsion will be released to destabilise the membrane's lipid bilayer leading to cell lysis and death

(Hamouda et al., 1999; Hamouda and Baker, 2000; Myc *et al.*, 2001; Hemmila *et al.*, 2010).

2.10 Analysis of bacterial stress in emulsions with flow cytometry

A flow cytometer consists of three main systems: the fluidics, optics, and electronics. The fluidics system directs the sample stream containing cells into a flowing stream of sheath fluid at laminar flow which aligns the sample stream to a one cell diameter in the direction of flow as they pass through an interrogation point and this is called hydrodynamic focusing. The optics system consists of a laser beam which illuminates one cell at a time at the interrogation point and optical filters to direct the light that emerges to the appropriate detectors (Figure 2.18A). The electronics system will then convert the detected light into electronic signals which is then processed by the computer (Givan, 2013).

At the interrogation point the laser light strikes one cell at a time resulting in the light being scattered at all angles. The amount of light scattered in the forward direction is collected by detectors that are typically up to 20° offset from the laser beam axis. The amount of light scattered to the sides is collected by detectors that are typically 90° offset from the laser beam axis. The forward and side-scattered lights are collected by detectors known as the forward scatter channel (FSC) and the side scatter channel (SSC), respectively (Figure 2.18B). The detectors produce electronic signals proportional to the optical signals reaching them. The signal intensity at the FSC is proportional to cell-surface area or size while at the SSC is proportional to granularity or structural complexity inside the cell (Givan, 2013).

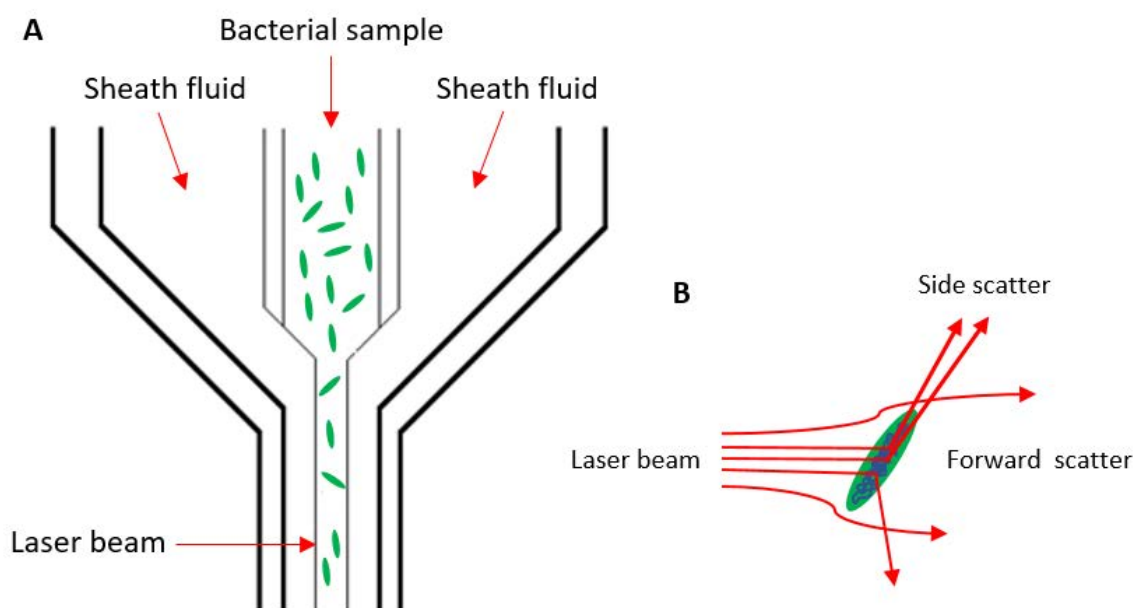


Figure 2.18 Flow cytometry. Hydrodynamically focused sample stream containing cells being transported to the interrogation point (A) and the laser light scattering properties of a cell (B).

Flow cytometry enables a near real-time analysis, quantification, and detection of bacterial cells in a population and that is an advantage over conventional techniques. When flow cytometry is used in conjunction with conventional techniques it becomes possible to enumerate stressed cells that cannot grow on plate. Flow cytometry enables multi-parametric single cell analysis which simultaneously measure multiple parameters and determine the physiological state of bacteria (Nebe-von-Caron *et al.*, 2000). An important feature in advanced flow cytometers is the ability to sort and isolate bacterial cells to very high purity which can then be used for further investigation (Jahan-Tigh *et al.*, 2012).

There are several fluorescent dyes that can be used in flow cytometry to analyse bacterial cells. The anionic lipophilic dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol or DiBAC₄(3) has been used in flow cytometry to quantify injured bacterial cells (López-Amorós *et al.*, 1995; Mason *et al.*, 1995; Deere *et al.*, 1995; Comas and

Vives-Rego, 1997; Amor *et al.*, 2002) as it has a high binding affinity for damaged membranes. Healthy bacterial cells will have a polarised membrane which excludes DiBAC₄ (3) as these cells are negatively charged in the interior. DiBAC₄(3) can only enter cells with a depolarised membrane where it binds to lipophilic charged molecules and upon excitation with a blue laser (488nm) the dye will emit green fluorescence (516nm) (Gatza *et al.*, 2012). The cationic dye propidium iodide (PI) has been used in flow cytometry to quantify dead bacterial cells (López-Amorós *et al.*, 1995; Grégori *et al.*, 2001; Amor *et al.*, 2002). Dead bacterial cells have compromised membrane. PI can only penetrate bacterial cells with a compromised membrane and bind to the nucleic acids and upon excitation with a blue laser (488nm) the dye will emit red fluorescence (636nm) (Gatza *et al.*, 2012). Both PI and DiBAC₄ (3) have been used in combination for multi-parametric single cell analysis in flow cytometry (López-Amorós *et al.*, 1995; Amor *et al.*, 2002).

The data in flow cytometry is represented as histograms and dot plots. On the histogram, the x-axis will present the relative fluorescence intensity while the y-axis presents the cumulative number of events (cell count). The histogram is composed of several adjacent rectangles aligned vertically each representing the cumulative number of events for a narrow range of relative fluorescent intensity. On a dot plot a single cell is shown as a dot and its position on the x-axis and y-axis will be determined according to the intensity detected for that cell. The computer can be configured to display only the fluorescent signals of bacterial cells with a specified set of scatter properties by allowing the user to 'gate' (specify an area on the plot) on any set of signals and this is known as scatter-gated fluorescence. It is also possible to

display two parameters a plot simultaneously and this is known as multiple parameter single cell analysis (Nebe-von-Caron *et al.*, 2000).

2.11 Double emulsion

The potential applications of $W_1/O/W_2$ emulsions expand to various industries including pharmaceutical, food, agricultural, and cosmetics. The potential applications of $W_1/O/W_2$ emulsions with emphasis on the microbiological applications will be discussed below.

2.11.1 Background of double emulsion

Since their first description in 1924 there has been increasing interest in $W_1/O/W_2$ emulsions due to their unique multi-compartmentalising structure that separates the inner W_1 droplets from the outer aqueous phase by a layer of another immiscible phase (Benichou *et al.*, 2004). Their ability to entrap and protect various hydrophilic substances and control their release from W_1 to W_2 made them applicable in various fields such as food, pharmaceuticals, and cosmetics. In the food industry $W_1/O/W_2$ emulsions can be used to make low calorie food products (Lobato-Calleros *et al.*, 2008), release of aroma and flavour (Malone *et al.*, 2003), salt reduction (Norton & Norton, 2010), food fortification by encapsulating vitamins (Benichou *et al.*, 2007; Li *et al.*, 2012), minerals (Bonnet *et al.*, 2009; Choi *et al.*, 2009), anti-microbial agents (Al-Nabulsi *et al.*, 2006), antioxidants (Maisuthisakul & Gordon, 2012), and amino acids (Owusu *et al.*, 1992). In the pharmaceutical industry $W_1/O/W_2$ emulsions have the potential for taste-masking to improve organoleptic properties of drugs (Chiappetta *et al.*, 2009), drug controlled release and targeted delivery of anti-cancer agents (Higashi and Setoguchi, 2000; Yanagie *et al.*, 2011), insulin to treat diabetes

(Cunha *et al.*, 1997) vaccine adjuvant to improve vaccine effectiveness (Bozkir *et al.*, 2004) and red blood substitute (Zheng and Zheng, 1993). The cosmetics industry is attempting to formulate creams (Laugel *et al.*, 1996) and soft capsules (Miyazawa *et al.*, 2000) to encapsulate compounds. Other applications of $W_1/O/W_2$ emulsions includes: the removal of toxic materials via entrapment and solubility enhancement of poorly-soluble materials (Yan & Pal, 2001), agriculture (Matsumoto *et al.*, 1976), aquaculture (Sato *et al.*, 1995), and separation processes (Larson *et al.*, 1994).

2.11.2 Segregation, protection, and delivery of bacteria in double emulsion

The encapsulation of microbial species in $W_1/O/W_2$ emulsion has been investigated for various applications. Shima *et al.* (2006) tested the effect of a model gastric juice on probiotic *Lactobacillus acidophilus* encapsulated in $W_1/O/W_2$ emulsion and as un-encapsulated cells. After 2-hour exposure to the low pH of the model gastric juice 49% of encapsulated cells were alive but no colonies were found with un-encapsulated cells. They concluded that the oil phase protects the *L. acidophilus* cells by preventing them from coming into contact with the model gastric juice. In a later study, they tested the effect of bile salts on encapsulated and un-encapsulated *L. acidophilus* cells and found an increase (~4-log higher) in survival of encapsulated compared to un-encapsulated cells (Shima *et al.*, 2009). Pimentel-González *et al.* (2009) found that after 2-hour exposure in a model gastric juice and bile salts the survival of *Lactobacillus rhamnosus* encapsulated in $W_1/O/W_2$ emulsion was higher than un-encapsulated cells. In another study Oaxaca cheese was made by incorporating $W_1/O/W_2$ emulsion containing probiotic *Lactobacillus plantarum* cells (Rodríguez-Huezo *et al.*, 2014). The effect of incorporation during cheese manufacture, melting and exposure to gastrointestinal conditions for 2 hours (low pH

and bile salts) on encapsulated and un-encapsulated *L. plantarum* cells was investigated. After cheese manufacture and melting the survival of encapsulated cells was higher (~2 and ~0.5-log higher, respectively) than un-encapsulated cells. After exposure to gastrointestinal conditions the survival of encapsulated cells was higher (~3-log higher, respectively) than un-encapsulated cells. Recently, $W_1/O/W_2$ emulsion was used to segregate two predominant species in soy sauce fermentation; *Zygosaccharomyces rouxii* (in W_1) and *Tetragenococcus halophilus* (in W_2) (Devanthi *et al.*, 2018). It was found that in model soy sauce fermentation the segregation of the two species using $W_1/O/W_2$ emulsion affected microbial cells growth and physiology, which led to the elimination of antagonism. These studies demonstrated the potential uses of $W_1/O/W_2$ emulsion as a delivery system for microbial species.

2.11.3 Double emulsions as bioreactors and tool for analysis

The internal W_1 environment can also be programmed dynamically without the need of rupturing the $W_1/O/W_2$ globules. The *E. coli* MC4100Z1 strain expresses GFP under anhydrotetracycline (aTC) dependent promoter and when present in the continuous phase, aTC can diffuse into core of droplets to activate GFP expression (Zhang *et al.*, 2013). Moreover, depending on the concentration of aTC in the continuous phase the expression of GFP can be controlled which was quantified using fluorescence microscopy. The authors suggest that the selectively-permeable oil barrier of the $W_1/O/W_2$ globules creates a discrete microenvironment whose aqueous core could be well defined and precisely modulated for cell cultivation, genetic activation and exploring complex biological processes such as bacteria quorum-sensing (Zhang *et al.*, 2013). $W_1/O/W_2$ emulsions have also been used to screen enzyme variants expressed in *E. coli* (Miller *et al.*, 2006). A method for rapid detection of

bacteriophages was developed by encapsulating *E. coli* with varying concentrations of lytic phages in $W_1/O/W_2$ emulsion globules and detection by fluorescence (Wang and Nitin, 2014). More recently $W_1/O/W_2$ emulsion globules have been used as 3D microenvironments for the containment and growth of bacterial biofilms (Chang *et al.*, 2015b). The *B. subtilis* 3610 strain was able to form 3D spherical biofilms on the inside of the oil shells detected by fluorescent microscopy using a dual-labelled reporter of the bacteria that expresses cyan fluorescent protein (CFP) when swimming and yellow fluorescent protein (YFP) when matrix forming. These studies present some of the important advancements for developing applications involving microbial encapsulation in $W_1/O/W_2$ emulsions.

2.11.4 Release of entrapped hydrophilic compounds in $W_1/O/W_2$ emulsions

A $W_1/O/W_2$ emulsion is an unstable system and oil globules will breakdown over time resulting in release of hydrophilic compounds from W_1 to W_2 . Deliberate destabilisation of $W_1/O/W_2$ emulsions if controlled can result in the release of hydrophilic compounds at the desired time. Controlling release of substances in $W_1/O/W_2$ emulsions may be useful in many applications. The release of hydrophilic compounds from the oil globules of $W_1/O/W_2$ emulsions can either involve the movement from W_1 to W_2 without oil globule breakdown or the liberation of the W_1 droplets and its contents into W_2 due to oil globule breakdown (e.g. bursting). Release mechanisms that do not involve breakdown of the oil globule includes surfactant-induced transport mechanisms and the coalescence of W_1 droplets with O/W_2 interface while oil globule breakdown results from the rupture of the interfacial film and can be shear stress-induced, thermally-induced, or osmotically-induced.

2.11.5 Release mechanisms not involving breakdown of oil globules

The mechanism of release of NaCl, halide salts, ephedrine hydrochloride and potassium nitrate from $W_1/O/W_2$ emulsions formulated as a potential drug delivery system was investigated (Sela *et al.*, 1995). The release mechanism was believed to be due to incorporation and transport via reverse micelles. It was suggested that the release occurred through three stages; the lag stage were the formation of reverse micelles and solubilisation of water in the reverse micelles occurs followed by fast release stage were the release is most rapid and finally the “no release” stage when equilibrium is reached due to a decrease in concentration difference between W_1 and W_2 . Fechner *et al.* (2007) investigated the release of vitamin B₁₂ from $W_1/O/W_2$ emulsions stabilised with two different hydrophilic surfactants: sodium caseinate (SC) and SC–dextran (Dex) conjugate and varying concentrations (2-8%) of PGPR during storage (12 weeks), acidification (pH 4) and heat treatment (90°C). The release of vitamin B₁₂ was significantly higher when the $W_1/O/W_2$ emulsion was stabilised with SC and 2% PGPR. The authors claim that the release was due to coalescence of W_1 droplets with the O/W_2 interface and that the SC-Dex conjugate provides a more stable interfacial film against coalescence. Weiss *et al.* (2005) used a combination of alginate and Ca²⁺ to gel the W_1 phase and investigated the release of the marker tryptophan from $W_1/O/W_2$ emulsions made with oils that have different fat content over storage for 24 hours at 23 or 7°C. They found that the release of tryptophan was lowest at 7°C especially when the fat content in the oil phase was increased. The authors suggest that the release of tryptophan was due to surfactant-induced mechanisms. Bonnet *et al.* (2009) investigated the release of magnesium from $W_1/O/W_2$ emulsions formulated with different types of oils based on their type of fatty

acid and viscosity over storage for 30 days at 4 or 25°C. The release of magnesium was highest at 25°C especially with oils of low viscosity and when the proportion of saturated fatty acids was high. The authors concluded that the release of magnesium was not due to coalescence between W_1 and O/W_2 interface but rather due to surfactant-induced mechanisms.

2.11.6 Release mechanisms involving breakdown of oil globules

Hattrem *et al.* (2014) studied the release of the synthetic dye tartrazine from $W_1/O/W_2$ emulsion stabilised with polysorbate 80 and varying concentrations of PGPR (0.4-2%) with or without a gelled W_1 phase (by adding gelatin to W_1) over storage for 90 days. The release of tartrazine was significantly higher when the concentration of PGPR was low. Also, the gelling of the W_1 phase prevented the release of tartrazine during the whole storage period. The authors suggested that the release of tartrazine was due to a surfactant-induced mechanism. However, when they added NaCl in the inner W_1 phase, swelling of the oil globules occurred due to osmotically driven water migration from W_2 to W_1 resulting in swelling-breakdown or bursting of the oil globules. They also found that the higher the concentration of NaCl in W_1 the higher the release of tartrazine.

Application of shear can also result in the breakdown of the oil globules and release of substances from W_1 and W_2 (Grossiord *et al.*, 2001). Olivieri *et al.* (2003) aimed to create a drug delivery system based on a $W_1/O/W_2$ emulsion that is shear sensitive at higher temperatures. By adding a thermally-reversible polymer (thickener) into W_2 the shear stress required to breakdown the oil globules become lower at a certain temperature. They investigated the release of NaCl from W_1 to W_2 at different shear

rates ($1000\text{--}5000\text{s}^{-1}$) and temperatures (20 or 35°C). At 35°C the complete release of NaCl was achieved at 2000s^{-1} while at 20°C the complete release of NaCl was achieved at 5000s^{-1} . In another study, volatile $W_1/O/W_2$ emulsions were produced for potential use as dermal delivery vehicles (Jaimes-Lizcano *et al.*, 2013). The oil phase consisted of cyclomethicones (silicon oils) with different volatilities and the evaporation of the $W_1/O/W_2$ emulsion was used as a trigger mechanism of releasing ingredients from W_1 to W_2 . Using fluorescence microscopy, the release of the fluorescently tagged bovine serum albumin (FITC-BSA) from W_1 was observed while the rate of evaporation was measured using thermo-gravimetric analysis. It was found that depending on the volatility of the oil the release rate can be controlled and the higher the volatility the faster the release rate.

When the oil globules of $W_1/O/W_2$ emulsion are in hyper-osmotic solution (e.g. adding electrolytes in W_2) and the osmotic difference between W_1 and W_2 is very large, the flux of water becomes so fast and immediate rupture of the globule is expected which leads to the delivery of the inner W_1 droplets into the continuous phase. Osmotic pressure difference that leads to oil globule breakdown was first observed when $W_1/O/W_2$ emulsion entrapping a drug was delivered *in-vivo* and immediate collapse of $W_1/O/W_2$ globules occurred due to the higher osmotic pressure of body fluids compared to the inner phase causing shrinking and/or bursting of the globules (Collings, 1971). On the other hand, when a $W_1/O/W_2$ emulsion is diluted in a hypo-osmotic solution the oil globules will swell until their breakdown or bursting occurs. The rate of swelling-breakdown or bursting of oil globules in $W_1/O/W_2$ emulsions diluted in hypo-osmotic solution was quantified by measuring the release of magnesium sulphate from W_1 to W_2 phase (Geiger *et al.*, 1998). The release was

reduced with increasing concentration of the lipophilic surfactant Abil® EM90 from 1 to 8%. The authors suggest that excess lipophilic surfactant in the oil phase increase the swelling capacity and therefore delay the bursting of the oil globules. Tedajo *et al.* (2005) investigated the swelling-breakdown release of chlorhexidine diglucoate (CHD) from oil globules of $W_1/O/W_2$ emulsions diluted (1:5, 1:10 and 1:20 dilution) in hypo-osmotic solution. The release was shown to be dependent on the dilution ratio and the higher the dilution ratio the higher the release of NaCl and CHD as a result of increased swelling-breakdown of the oil globules. Cárdenas and Castro (2013) investigated the effect of changing the concentration of W_1 (30, 50 and 60%) and NaCl in W_1 (0.5, 2.5 and 10%) on the swelling-breakdown of the oil globules in $W_1/O/W_2$ emulsion quantified by measuring the release of NaCl from W_1 to W_2 . They found that the bursting of oil globules was higher when the concentration of W_1 and NaCl was highest (60 and 10%, respectively). Using fluorescence capillary video-microscopy the release of FITC-BSA from $W_1/O/W_2$ emulsions was investigated after a freeze-thaw cycle (Rojas *et al.*, 2008). After thawing the coalescence between W_1 and O/W_2 interface occurs resulting in the release of the FITC-BSA. During freezing the surfactant molecules at the surface of the oil globules are expelled due to their lower solubility in the solid phase and this trigger bursting of the oil globules during thawing as the oil soluble surfactant has not yet fully moved to the surface of the O/W_2 interface.

CHAPTER 3: EFFECT OF OIL-IN-WATER (O/W) NANO-EMULSIONS ON SURVIVAL AND GROWTH OF BACTERIA

3.1 Introduction

Nano-emulsions gained popularity in food production due to improving food properties and formulations, for example, use of less fat and emulsifiers, increased emulsion stability and improved optical appearance, enhancement of taste and sensory perception of ingredients or masking of certain ingredients (Chaudhry and Castle, 2011). Nano-emulsion manufacturing requires more energy than emulsions with smaller droplet sizes (Gupta *et al.*, 2016) and they possess different physicochemical properties to coarse emulsions (McClements, 2010) due to their nano-sized droplets (Baglioni and Chelazzi, 2013) and increased interface. Nano-emulsions have shown antimicrobial activity against a variety of Gram-positive and Gram-negative bacteria including *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus megaterium*, *Bacillus subtilis* and *Bacillus circulans* (Hamouda *et al.*, 1999; Baker *et al.*, 2000; Teixeira *et al.*, 2007; Bharghava *et al.* 2015; Jo *et al.*, 2015; Majeed *et al.*, 2016; Lu *et al.*, 2017). Furthermore, nano-emulsions were found to selectively disrupt the membrane of prokaryotic cells but not eukaryotic cells (Baker *et al.*, 2000), which could expand their applications in managing safety and microbial growth in food through formulation. The antimicrobial effect of nano-emulsions has been attributed to their structure itself and the nano-sized droplets. When nano-emulsions are formed under high shearing forces (e.g. ultrasonication, high-pressure homogenisation or high-shear mixing) they acquire

significant amount of energy as they are formed (Lee *et al.*, 2010). The nano-droplets are thermodynamically driven to fuse with lipid-containing micro-organisms and the energy that was stored during formation of the nano-emulsion is will be released to destabilise the membrane's lipid bilayer leading to cell lysis and death (Hamouda *et al.*, 1999; Hamouda and Baker, 2000; Myc *et al.*, 2001; Hemmila *et al.*, 2010).

However, after summarising and reviewing the literature discussing the antimicrobial activity of nano-emulsions (Table 3.1), there is evidence of contradiction and no consistency of effect on the same species of bacteria. For example, two studies found no correlation between droplet size and antimicrobial activity (Buranasuksombat *et al.*, 2011; Terjung *et al.*, 2012). Buranasuksombat *et al.* (2011) found that nano-emulsions (<300 nm droplet size) made from soybean oil and the non-ionic surfactant Tween80 had no antimicrobial effects on *E. coli*, *S. typhimurium*, *L. monocytogenes*, *B. cereus* and *P. aeruginosa* after exposure for 30 minutes, unless the oil phase itself contained antimicrobial properties. Terjung *et al.* (2012) found that the antimicrobial properties of nano-emulsion (80 nm droplet size) made from Miglyol 812N and Tween80 were less effective in inhibiting growth of *E. coli* and *Listeria innocua* compared to coarse emulsion (3 µm). Therefore, more work is required to confirm with confidence antimicrobial activity of nano-emulsions, exclusive to structure and droplet size. In other cases (Table 3.1) the antimicrobial activity was investigated in nano-emulsions containing antimicrobial components which were either added in the formulation or were natural components of the oil. These studies concluded that nano-emulsions enhanced the activity of the antimicrobial component; surprisingly, the controls in place were not appropriate for

supporting the conclusions, and antimicrobial activity was attributed to nano-emulsion structure instead of the formulation and the antimicrobial component.

The aim of this study was to comprehensively assess the effect of a model O/W nano-emulsion on bacteria, specifically, microbial survival in minimal growth medium at ambient temperature (M9 medium at 25°C), microbial growth in rich medium (30°C), and cell membrane integrity by flow cytometric analysis. As the O/W emulsion structure can be affected by the interaction of bacterial cell properties with the emulsion interface (Ly *et al.*, 2006; Ly *et al.*, 2008), the study included different Gram-negative and Gram-positive bacterial species and strains of varying surface charge, hydrophobicity and ability to form the protein adhesin curli. Finally, in order to investigate the effect of O/W emulsion structure, i.e. size of the oil droplets, in combination with antimicrobial components in formulation, caprylic acid (CA) was added in the oil phase. CA is an eight-carbon short-chain fatty acid found naturally in milk with well documented antimicrobial activity in bulk against various microbial species (Nair *et al.*, 2005; Annamalai *et al.*, 2000; Andrews *et al.*, 2001), however, no study has yet assessed CA as part of an emulsion formulation. Since CA is minimally soluble in water and due to its fat solubility, it can be incorporated within the oil phase of O/W nano-emulsions, and highlights possible increases in antimicrobial activity due to increase in interface. Changes in the stability of O/W nano-emulsions in the presence of bacteria were monitored by measuring the droplet size and creaming height while fluorescence microscopy was employed to screen the localisation and distribution of bacteria within the emulsions.

Table 3.1 The antimicrobial activity of nano-emulsions correlated with their mean droplet size, ingredients (oil phase, stabilisers, and antimicrobials) and the micro-organism it is tested against. The impact of the nano-emulsion is classified as positive (+) when the nano-sized droplets improves the antimicrobial activity with respect to the control, as negative (–) when it decreases the antimicrobial activity, and as neutral (+/–) when no significant change is observed or a significant change was observed only due to incorporation of antimicrobials and not because of nano-size droplets. The method of emulsification is mentioned as: HPH – High Pressure Homogenisation, HSH – High Shear Homogenisation, US – Ultrasonication, MFZ – Microfluidizer, CPI – Catastrophic Phase Inversion.

Emulsion type	Oil phase	Stabilisers	Continuous phase	Antimicrobials	Mean droplet size (nm)	Method of emulsification	Impact of nano-emulsion	Micro-organism	Controls	Author
O/W nano-emulsion	Euganol 5-12.5% (w/w)	Tween20	Water	None	50–110nm	US	+	<i>Fusarium oxysporum f. sp. vasinfectum</i>	Untreated sample	Abd-Elsalam <i>et al.</i> (2015)
O/W emulsion	Oregano oil 0.05 or 0.1% (w/w)	Tween80	Water	None	148nm	US	+	<i>L. monocytogenes</i> <i>S. Typhimurium</i> <i>E. coli</i> O157:H7	Water	Bhargava <i>et al.</i> (2015)
O/W nano-emulsion	Grindsted Acetem 90-50K 10-15% (w/w)	Tween60	Water	Cinnamaldehyde 3-10% (w/w)	79±2nm	HPH	-	<i>L. monocytogenes</i> <i>E. coli</i> O157:H7	Nano-emulsion without cinnamaldehyde	Bilbao-Sainz <i>et al.</i> (2013)
O/W nano-emulsion	LMO Lemon myrtle 5% (w/w) SBO Soybean oil 16% (w/w) BCTP Soybean oil 16%	LMO Tween 80 SBO Tween80 BCTP Triton X-100,	Water	None	97±2nm	MFZ	+/-	<i>E. coli</i> <i>L. monocytogenes</i> <i>S. Typhimurium</i> <i>P. aeruginosa</i> <i>B. cereus</i>	Coarse emulsion	Buranasuksombat <i>et al.</i> (2011)

	(w/w)	tributyl- <i>n</i> -phosphate								
O/W nano-emulsion	Thyme and corn oil 10% (w/w)	Tween 80 or Tween and LAE	Water	None	<200nm	HPH	+	<i>Z. bailii</i>	Thyme or corn oil nano-emulsion with no LAE	Chang <i>et al.</i> (2015)
O/W nano-emulsion	Thyme oil and corn oil or MCT medium chain triglyceride (MCT) (from 0 to 100% (w/w)) (10% (w/w))	Tween 80	Aqueous buffer solution (5mM citrate buffer, pH 3.5)	None	160-196nm	HPH	+	<i>Zygosaccharomyces bailii</i>	Nano-emulsion with corn or MCT but no thyme oil	Chang <i>et al.</i> (2012)
O/W nano-emulsion	D-Limonene 5% (w/w) or a mixture of terpenes 5% (w/w)	Soy lecithin Solec Ip, Tween 20 and glycerol monooleate and CLEARGUM CO 01	Water	D-limonene and a mixture of terpenes extracted from <i>Melaleuca alternifolia</i> (0.1-10% (w/w))	74.4-156.6nm	HPH	+	<i>E. coli</i> <i>L. delbrueckii</i> <i>S. cerevisiae</i>	Sunflower oil with D-limonene (50:50) 10% (w/w) or Palm oil with a mixture of terpenes (50:50) 10% (w/w)	Donsi <i>et al.</i> (2011)
O/W nano-emulsion	Sunflower oil 8% (w/w)	Lecithin, pea proteins, sugar ester, and a combination of Tween20 and glycerol monooleate	Water	Carvacrol, D-limonene and cinnamaldehyde 2% (w/w)	170-240nm	HPH	+	<i>E. coli</i> <i>L. delbrueckii</i> <i>S. cerevisiae</i>	Water	Donsi <i>et al.</i> (2012)
O/W nano-emulsion	BCTP Soybean oil 16% (v/v) BCTP-CPC Soybean oil 16% (v/v) TEOP Ethyl oleate 3% (v/v)	BCTP Tri- <i>n</i> -butyl phosphate, and Triton X-100 BCTP-CPC Tri- <i>n</i> -butyl phosphate, and Triton X-100 TEOP <i>n</i> -pentanol and Tween80	Water	BCTP Water BCTP-CPC CPC 0.25% (w/v) TEOP None	N.A.	US	+/-	<i>S. aureus</i> <i>E. coli</i> <i>L. monocytogenes</i>	Water, CPC solution 0.25% (w/v), tributyl phosphate solution, bulk soybean oil, Triton X-100, Tween80 or <i>n</i> -pentanol solution	Ferriera <i>et al.</i> (2010)
O/W nano-emulsion	Basil oil 6% (v/v)	Tween80	Water	None	29.3nm	US	+	<i>E. coli</i>	PBS	Ghosh <i>et al.</i>

										(2013)
O/W nano-emulsion	Mustard oil 6% (v/v)	Tween20	Water	None	18-430nm	Magnetic stirrer	+	<i>E. coli</i>	Untreated sample	Ghosh <i>et al.</i> (2012)
O/W nano-emulsion	Sesame oil 6% (v/v)	Tween20 or Tween80	Water	Euganol 1-6% (v/v)	20nm	US	+/-	<i>S. aureus</i>	PBS or sodium benzoate 0.3% (v/v)	Ghosh <i>et al.</i> (2014)
W/O nano-emulsion	BCTP Soybean oil 80% (w/w) BCTP 401 Soybean and peppermint oil 80% (w/w)	BCTP Tri- <i>n</i> -butyl phosphate and Triton X-100 BCTP 401 Tri- <i>n</i> - butyl phosphate, Triton X-100, glycerol monostearate, refined soya sterols, Tween60	Water	CPC	400-800nm	N.A.	+	<i>B. cereus</i> spores <i>B. circularis</i> spores <i>B. megaterium</i> spores <i>B. subtilis</i> spores	Different dilutions of BCTP and BCTP 401 (1:10, 1:100 and 1:1000)	Hamoud a <i>et al.</i> (1999)
W/O nano-emulsion	8N8 Soybean oil 64% (w/w) W60C Soybean oil 20% (w/w)	8N8 Tri- <i>z</i> -butyl phosphate and Triton X-100 W60C Tween60, glycerol monooleate and refined soya sterols	Water	8N8 None W60C CPC 1% (w/w)	400-800nm	N.A.	+	<i>E. coli</i> <i>Vibrio cholerae</i> <i>S. typhimurium</i>	Tris-EDTA buffer solution	Hamoud a & Baker (2000)
O/W nano-emulsion	Soybean oil (%N.A.)	Ethylenediamine, acetic acid, glycerol, Tween20, and benzalkonium chloride	Saline solution 0.9% (w/w)	None	350nm	HSH	+	<i>P. aeruginosa</i>	Saline solution 0.9% (w/w)	Hemmila <i>et al.</i> (2010)
O/W nano-emulsion	Soybean oil 25% (v/v)	Triton X-100	Water	CPC 1% (v/v)	100-800nm	MFZ	+	<i>Acinetobacter baumannii</i>	Ethanol 30% (v/v) or untreated sample	Hwang <i>et al.</i> (2013)
O/W micro-emulsion	Micelles of Tween20 0.6% (w/w)	Tween20	Water	Trans-cinnamaldehyde 0.2% (w/w)	127nm	HPH	+	<i>S. Typhimurium</i> <i>S. aureus</i> <i>E. coli</i> O157:H7	Water or Watermelon juice	Jo <i>et al.</i> (2015)
O/W nano-emulsion	Soybean oil 25% v/v)	Triton X-100 10%	Water	CPC 1% (w/v)	308nm	MFZ	+	<i>S. mutans</i>	Chlorhexidine	Karthike

		(v/v)			Microfluidizer (M-110L, Microfluidics, Newton, MA) at 20,000 psi			(planktonic and biofilm)	digluconate 0.12% (v/v) or untreated sample	yan <i>et al.</i> (2011)
O/W nano-emulsion	Soybean oil 25% (v/v)	Triton X-100 10% (v/v)	Water	CPC 1% (w/v)	308nm Microfluidizer (M-110L, Microfluidics, Newton, MA) at 20,000 psi for	MFZ	+	<i>S. mutans</i> (planktonic and biofilm) <i>L. casei</i> (planktonic and biofilm) <i>Actinomyces viscosus</i> <i>Candida albicans</i>	Chlorhexidine digluconate 0.12% (v/v) or untreated sample	Karthikeyan <i>et al.</i> (2012)
O/W nano-emulsion	Lemongrass oil 0.5-4% (w/w)	Tween80 0.1, 0.5, 0.75 and 1% (w/w)	Water	None	56.5-87.6nm	HPH	+	<i>S. typhimurium</i> <i>E. coli</i> O157:H7	Untreated sample	Kim <i>et al.</i> (2013)
O/W nano-emulsion	Pure peppermint oil, medium chain triglyceride (MCT), and their mixture at ratios of 1:5, 1:1, and 5:1 (v/v)	Modified starch	Water	None	184-228nm	HPH	+	<i>L. monocytogenes</i> <i>S. aureus</i>	Bulk peppermint oil, MCT nano-emulsion or untreated sample	Liang <i>et al.</i> (2012)
O/W nano-emulsion	Citral oil 10% (w/w) Surfactants:	Span 85, Brij 97 and ethylene glycol	Water	None	28nm	US	+	<i>S. aureus</i> <i>E. coli</i> <i>Pseudomonas aeruginosa</i> <i>Enterococcus faecalis</i> <i>S. typhimurium</i> <i>L. monocytogenes</i>	Sulphadiazine	Lu <i>et al.</i> (2017)
O/W nano-emulsion	Soybean oil 25% (v/v)	Triton X-100 10% (v/v)	Water	CPC 1% (w/v)	168 nm	N.A.	+	<i>S. mutans</i> <i>L. casei</i>	Chlorhexidine gluconate 0.12% (v/v) or untreated sample	Lee <i>et al.</i> (2010)

O/W emulsion	Clove or canola oil 10% (v/v) and a mixture at ratios of 1:9, 3:7 and 5:5 10% (v/v)	Tween80 and modified starch	Water	None	151.3-203.9nm	HPH	+/-	<i>L. monocytogenes</i> <i>S. aureus</i> <i>E. coli</i>	Nano-emulsion with canola oil with no clove oil	Majeed <i>et al.</i> (2016)
O/W nano-emulsion	<i>Thymus daenensis</i> oil 2% (w/w)	Tween80 and lecithin	Water	None	143nm	US	+	<i>E. coli</i>	Bulk <i>Thymus daenensis</i> oil or untreated sample	Moghim <i>et al.</i> 2016a
O/W nano-emulsion	Sage oil (<i>Salvia officinalis</i>) 20% (w/w)	Tween80 and Span80	Water	None	222nm	US	+	<i>E. coli</i> <i>S. dysentery</i> <i>S. typhi</i>	Bulk sage oil or untreated sample	Moghim <i>et al.</i> 2016b
W/O nano-emulsion	X8W60PC Oil 64% (w/w)	Three non-ionic detergents and solvent	Water	None	400–800nm	HSH	+	<i>Candida parapsilosis</i> <i>Fusarium oxysporum</i> <i>Candida albicans</i> <i>Candida tropicalis</i> <i>Microsporum gypseum</i> , <i>Trichophyton mentagrophytes</i> <i>Trichophyton rubrum</i> , and <i>Aspergillus fumigatus</i>	Untreated sample or bleach 6%	Myc <i>et al.</i> (2001)
O/W micro-emulsion	Micelles 1, 2, 3, 5, 7.5, and 10.0% (w/v)	Surfynol 485W or SDS or Tween20 or CG20 contains LAE 10% (w/v)	Water	Eugenol or carvacrol 0.01% to 8.0% (w/v)	Not mentioned	Magnetic stirrer	+	<i>E. coli</i> O157:H7 <i>S. enterica</i> serotype	Water	Ruengvi sesh <i>et al.</i> (2015)
O/W nano-emulsion	Lemongrass oil 1% (v/v)	Tween80	Sodium alginate 1% (w/v)	None	4-35nm	MFZ	+	<i>E. coli</i>	Water	Salvia-Trujillo <i>et al.</i> (2015)

O/W nano-emulsion	Sunflower oil 2-3% (w/w)	Tween20 and glycerol monooleate	Water	Carvacrol 2% (w/w), bergamot 3% (w/w), mandarin 3% (w/w) and lemon essential oils 3% (w/w)	133.4-176.4nm	HPH	+	<i>E. coli</i> O157:H7 <i>S. Typhimurium</i>	Untreated sample	Severino <i>et al.</i> (2015)
O/W nano-emulsion	Sunflower oil 2% (w/w)	Tween 20 and glycerol monooleate	Water	Mandarin essential oil 2% (w/w)	176.4 ± 14.5 nm	HPH	+	<i>L. innocua</i>	Untreated sample	Severino <i>et al.</i> (2014a)
O/W nano-emulsion	Sunflower oil 2-3% (w/w)	Tween 20 and glycerol monooleate	Water	Carvacrol 1% (w/w), bergamot 2% (w/w), mandarin 2% (w/w) and lemon essential oils 2% (w/w)	133.4-176.4nm	HPH	+	<i>L. monocytogenes</i> (5 strains)	Untreated sample	Severino <i>et al.</i> (2014b)
O/W nano-emulsion	Lemon, mandarin, oregano or clove essential oils 5% (w/w)	Glycerol monooleate or soy lecithin, whey protein isolate, pea proteins, Tween 20	Water	None	88-394nm	HPH	+	Endogenous flora of Rucola leaves	Untreated sample	Sessa <i>et al.</i> (2015)
O/W nano-emulsion	Hexane 10% (v/v)	Whey protein isolate	Water	Euganol 2% (v/v)	127-255 nm	HSH	-	<i>E. coli</i> O157:H7 <i>L. monocytogenes</i> Scott A	Untreated sample or eugenol (4.5 g/L) 2% reduced fat milk	Shah <i>et al.</i> (2013)
O/W nano-emulsion	Eucalyptus oil 16.66% (v/v)	Tween80	Water	None	17.1nm	US	+	<i>B. cereus</i> <i>S. aureus</i> <i>E. coli</i>	Bulk eucalyptus oil or water containing Tween80 16.66% (v/v)	Sugumar <i>et al.</i> (2013)
O/W nano-emulsion	Eucalyptus oil 16.66% (v/v)	Triton X-100	Water	None	3.8nm	US	+	<i>S. aureus</i>	Untreated sample	Sugumar <i>et al.</i> (2014)
O/W nano-emulsion	BCTP Ethyl oleate 3% (v/v)	BCTP <i>n</i> -pentanol and	Water	None	Not mentioned	HSH	+	<i>S. typhimurium</i>	Untreated sample	Teixiera <i>et al.</i>

	TEOP Soybean oil 16% (v/v)	TEOP Tri- <i>n</i> -butyl phosphate 2% (v/v), and triton X-100 2% (v/v)						<i>E. coli</i> O157:H7 <i>Pseudomonas aeruginosa</i> <i>S. aureus</i> <i>L. monocytogenes</i>		(2007)
O/W nano-emulsion	Miglyol 812N 10% (w/w)	Tween80	Water	Carvacrol and eugenol (5, 15, 30 and 50 (w/w %))	80nm	HPH	-	<i>E. coli</i> C 600 <i>Listeria Innocua</i>	Coarse emulsion	Terjung <i>et al.</i> (2012)
O/W nano-emulsion	Anise oil 75% (w/w)	Alcolec PC75 (soy lecithin)	Water	None	117.2–275.7nm	HPH	+	<i>L. monocytogenes</i> <i>E. coli</i> O157:H7	Anise bulk oil or coarse emulsion	Topuz <i>et al.</i> (2016)
O/W nano-emulsion	Thyme oil 1% (w/w)	Propylene glycol and 1% sodium dodecyl sulfate	Water	None	279nm	HSH	+	<i>L. monocytogenes</i> Scott A <i>S. Enteritidis</i> <i>E. coli</i> O157:H7	Bulk thyme oil	Wu <i>et al.</i> (2014)
O/W nano-emulsion	Thyme 1% (w/v)	Sodium caseinate and lecithin	Water	None	82.5-125.5nm	HSH	+	<i>E. coli</i> O157:H7 <i>S. enterica serovar Enteritidis</i> <i>L. monocytogenes</i> Scott A	Bulk thyme oil or a mixture of water and milk	Xue <i>et al.</i> (2015)
O/W micro-emulsion	<i>Laurus nobilis</i> essential oil 15% (w/v)	Tween20 and ethanol	Water	None	10nm	N.A.	+	<i>Alternaria alternata</i>	Water	Xu <i>et al.</i> (2017)
O/W nano-emulsion	D-limonene 4% (w/w)	Propylene glycol and Tween80	Water	Nisin 0%, 0.5, 1.5 or 3.0% (w/w)	16.34nm-18.92nm	CPI	+	<i>S. aureus</i> <i>B. subtilis</i> <i>E. coli</i> <i>S. cerevisiae</i>	Nutrient (bacteria), YPD broth (yeast) or of kanamycin sulphate (50 lg/ml) in broth	Zhang <i>et al.</i> (2014)
O/W nano-emulsion	Thyme oil and corn oil (from 0 to 100% (w/w) corn oil 5% (w/w)	Tween80 and lauric arginate (LAE) or sodium dodecyl sulfate (SDS)	Buffer solution (10mM acetate, pH 4)	None	163nm	HPH	+	<i>Zygosaccharomyces bailli</i> <i>Saccharomyces cerevisiae</i> <i>Brettanomyces</i>	Buffer solution (10mM acetate, pH 4)	Ziaini <i>et al.</i> (2011)

								<i>bruxellensis</i>		
								<i>Brettanomyces</i>		
								<i>naardenensis</i>		

3.2 Materials and Methods

3.2.1 Microbial cultures

The water-soluble emulsifier polysorbate 80 (Tween80), hexane 95% and caprylic acid (CA) $\geq 98\%$ were purchased from Sigma-Aldrich (United Kingdom). Sunflower oil (food grade) was purchased from a local retailer (United Kingdom). Nucleic acid stains 2-(4-amidinophenyl)-1H-indole-6-carboxamide (DAPI) and propidium iodide (PI) were purchased from Sigma-Aldrich (United Kingdom). Tryptic soy agar (Oxoid Ltd. CM0131), tryptic soy broth (Oxoid Ltd. CM0129), nutrient agar (Oxoid Ltd. CM0003), *de Man, Rogosa and Sharpe* (M.R.S) agar (OXOID CM0359) and broth (OXOID CM0361) were purchased from Fisher Scientific (United Kingdom).

3.2.2 Microbial cultures

Escherichia coli K-12 strains MG1655 (CGSC 6300), BW25113 (CGSC 7636), JM109 (NEB E4107), MC4100 (CGSC 6152) and its derivative PHL644 (MC4100 *malA-kan ompR234*) (Vidal *et al.* 1998) were maintained on tryptic soy agar at 4°C. *Bacillus cereus* (NCTC 11143), and *Staphylococcus epidermidis* (NCIMB 10387) were maintained on nutrient agar at 4°C. *Lactobacillus acidophilus* (ATCC 4356) was maintained on M.R.S agar at 4°C. For obtaining cells in the exponential phase, cells were harvested from growth medium by centrifugation (10,000 g, 10 minutes) and washed in PBS (phosphate buffered saline) solution twice. *E. coli*, *B. cereus*, and *S. epidermidis* cells were each transferred into 50 ml of tryptic soy broth, incubated at 37°C for 24 hours shaking at 150 rpm and sub-cultured to 50 ml of tryptic soy broth for a further 2 hours (*E. coli*) or 4 hours (*B. cereus* and *S. epidermidis*). *L. acidophilus* cells were transferred into 50 ml of M.R.S broth,

incubated at 37°C for 42 hours and sub-cultured to 50 ml of M.R.S broth for a further 12 hours.

3.2.3 Bacterial-adhesion-to-hydrocarbon (BATH) test

The hydrophobicity of bacterial cell surfaces was evaluated as by bacterial-adhesion-to-hydrocarbon (BATH) according to the method proposed by Rosenberg *et al.* (1980). The optical density (A_o) of bacterial cells ($\sim 10^9$ CFU/ml) harvested in the exponential phase by centrifugation (10,000 g, 10 minutes) and washed twice in *PBS* and re-suspended in M9 medium was measured at 600nm. Four millilitres of the bacterial suspension were mixed with 1 ml hexane by vortexing for 2 minutes and then left to stand for 15 min to allow separation of layers, at which time the optical density at 600 nm (A_t) was again measured by carefully removing a sample (1ml) from the aqueous phase. The percentage of bacterial adhesion to hexane was expressed by the difference of the absorbance of cell suspension before (A_o) and after (A_t) mixing with the solvent: $(1 - A_t/A_o) \times 100$. The percentage of bound cells was subsequently calculated by % adherence = $(1 - A_t/A_o) \times 100$ where A_o is the optical density measured at 600 nm of the bacterial suspension before mixing and A_t is the absorbance after mixing. The mean percentage of partitioning of an organism into the hexane phase was calculated by using triplicate samples.

3.2.4 ζ -potential (zeta potential) measurements

For measuring the ζ -potential of bacteria, cells were harvested in exponential phase by centrifugation, washed twice in *PBS*, re-suspended and diluted in M9 medium to a density of 10^7 cells per ml. One millilitre of the samples was injected in a universal folded capillary cell (Model DTS 1070, Malvern Instruments Ltd, UK) equipped with platinum electrodes and a folded capillary, checking that all air bubbles were

removed. The electrophoretic mobility (EM) at 150V of the suspended bacteria was then measured at 25°C using Malvern ZetaSizer Nano ZS (Malvern Instruments Ltd, UK), which uses the scattering of incident laser light to detect the bacteria at relative low magnification. The instrument was calibrated using the ζ -potential transfer standard (DTS1235) which has a ζ -potential of $-42\text{mV} \pm 4.2\text{mV}$. The mobility of the bacteria under the applied voltage was converted to the ζ -potential using the Smoluchowski equation and reported as the average and standard deviation of measurements made on two freshly prepared samples, with three readings made per sample. For measuring the ζ -potential of single O/W emulsions, freshly made O/W emulsions were diluted 1:10 in M9 media and one millilitre of the diluted emulsions were then injected in a universal folded capillary cell and the ζ -potential was measured as previous.

3.2.5 Preparation of O/W emulsions

Coarse O/W emulsions were prepared using a high shear mixer homogeniser (Silverson L5M) at 25°C. The continuous phase was prepared by dissolving Tween80 (8 wt%) in tryptic soy broth or M9 media at 60°C for 15 minutes. Nano-emulsions were prepared by homogenising sunflower oil in the continuous phase at 5000 rpm for 60 seconds and the homogenised emulsions were sonicated with a probe sonicator (VCX 750 Sonics, USA) using a 22mm horn tip and operating at a frequency of 20 kHz and 750 watts for 4 minutes. Control coarse emulsions were prepared by homogenising sunflower oil in the continuous phase (ratio of 40:60 or 20:80) at 3000 rpm for 60 seconds. For microbial viability studies, bacterial cells ($\sim 10^8$ CFU/ml) were washed twice and re-suspended in ten millilitres O/W emulsions (M9 media as continuous phase) or 6 ml M9 minimal growth medium (control) and incubated at 25°C for 2 and 7 days on a rotator (Stuart SB3, UK) at 2 rpm to ensure

homogenised mixing. For growth studies, bacterial cells ($\sim 10^4$ CFU/ml) were washed twice in *PBS* and re-suspended in ten millilitres of O/W emulsions (tryptic soy broth as continuous phase) or 6 ml of tryptic soy broth (control) and inoculated with and incubated at 30°C over time on a rotator at 2 rpm.

In order to investigate the effect of oil droplet size in O/W emulsions in combination with antimicrobial components, caprylic acid (CA) was added in the oil phase. Since CA is minimally soluble in water and soluble in fat, its effect should be affected by the surface area of the oil phase. Bacterial cells ($\sim 10^8$ CFU/ml) were washed twice and re-suspended in ten millilitres O/W emulsions (M9 media as continuous phase) or 8 ml M9 minimal growth medium with 2 ml bulk oil with (0.5 or 1% CA) or without CA and incubated at 25°C for 1, 8 and 24 hours on a rotator at 2 rpm.

3.2.6 Characterisation of emulsion stability during incubation

3.2.6.1 Measurement of oil globule size [D (4, 3)]

The particle size distribution of the oil globules was measured immediately after preparation and as a function of storage time using a laser diffraction particle size analyser (Malvern Mastersizer 2000, Malvern Instrument Ltd, Worcestershire, UK), equipped with a He-Ne laser ($\lambda = 633\text{nm}$). The dispersion unit stirring speed was kept at 2000 rpm and the measurement range was 0.02–2000 μm . The optical parameters selected were: dispersed phase refractive index of n_D^{22} 1.39; oil globule absorbance of 0.01; and a dispersant liquid (distilled water) refractive index n_D^{22} 1.33; obscuration between 10% and 20%. At refractive of n_D^{22} 1.39 *E. coli* cells are not detected which ensures only the oil droplets are being measured. To ensure homogenised mixing prior to measurements the sample was mixed by gently by

hand and was added dropwise to the system until the obscuration was within an acceptable range. Particle size calculations were based on the Mie Scattering theory and the volume mean diameter values ($D_{[4, 3]}$), and the percentage of volume corresponding to each observed population were calculated using the Mastersizer 2000 software.

3.2.6.2 Observation of phase separation

The cream height fraction of the coarse emulsion was measured immediately after preparation and as a function of storage time. Five millilitres of O/W emulsion were transferred to a graduated 10ml centrifuge tube and left standing upright for 1 hour. The appearance of a cream layer was observed and the cream height fraction was visually measured at 1-hour from the time creaming started. The expression used for calculation of the creaming percentage height is as follows:

$$H_{\text{cream}} = ((H_{\text{emulsion}} - H_{\text{serum}}) / H_{\text{emulsion}}) \times 100\% \quad (3.1)$$

3.2.7 Determination of bacterial cell viability and growth

Serial dilutions in *PBS* and plating on tryptic soy agar using the Miles & Misra technique (Miles *et al.*, 1938) was conducted immediately after preparation and as a function of storage time to obtain bacterial cell counts as colony forming units per millilitre (CFU/ml).

3.2.8 Flow cytometric analysis of bacterial cells

Flow cytometric analysis was conducted immediately after preparation and as a function of storage time using a BD Accuri C6 flow cytometer (BD, Oxford, UK). From a 1 millilitre sample, the bacterial cells were harvested by centrifugation by centrifugation (10,000 g, 10 minutes) and washed twice and re-suspended in *PBS*.

The bacterial cells were stained by adding PI (4 μ l/ml) and incubated in the dark for 30 minutes. Samples were excited using a 488nm solid state laser and particulate noise was eliminated using a Forward scatter height (FSC-H) threshold while 20,000 data points were collected at a maximum rate of 2500 events/s. Fluorescence was detected using 670 LP filters corresponding to PI fluorescence. The data was analysed using CFlow (BD).

3.2.9 Fluorescent and optical imaging of bacteria in O/W emulsions

The O/W emulsions with bacteria were observed using optical and fluorescent microscopy (Zeiss Axioplan) at ambient temperature. The sample was stained by adding DAPI (4 μ l/ml) and incubated in the dark for 30 minutes. The stained sample was placed on a microscope slide and gently covered with a cover slip. The images were acquired under objective lens 100x magnification (oil immersion) with a digital camera system AxioCam ICm1 using a 1.4 megapixel monochrome CCD camera via AxioVision Software (Zeiss). The samples were observed at room temperature using a fluorescent microscope (Zeiss AxioLab) equipped with a mercury arc lamp and the emission was observed at 461nm (DAPI). Micrographs were overlaid using analysis software (ImageJ).

3.2.10 Statistical analysis

Each experiment was conducted at least in duplicate (N=2) and some cases in triplicate (N=3). The generated results were collected in Excel (Microsoft Corp.) for calculating means, standard deviations and error bars. For Student's *t*-test to compare two means or one-way analysis of variance (ANOVA) and the Tukey's HSD *post hoc* test to compare several means were used for checking whether there is

significant difference among samples using IBM SPSS Statistics software version 21. Differences were considered significant at $P < 0.05$.

3.3 Results

3.3.1 Characterisation of the O/W emulsions

The O/W emulsion formulations were characterised in terms of oil droplet size [(D (4, 3)]. Two types of O/W emulsions with different D (4, 3) were achieved depending on the formulation: coarse emulsions (15-35 μ m) and nano-emulsions (170-650nm) (see Appendix, Fig. A1).

3.3.2 ζ -potential and hydrophobicity

To understand whether the bacteria possess different physicochemical surface properties that can affect the stability of the O/W emulsions the bacteria were characterised in terms of their cell surface hydrophobicity and ζ -potential (Table 2.1). All the bacteria had negative ζ -potentials. From the Gram-negative bacteria, *E. coli* (JM109) and *E. coli* (PHL644) were the most hydrophobic with %adhesion of 10.10 ± 3.70 and 4.10 ± 2.00 , respectively, and had the most negative ζ -potential with -16.63 ± 0.11 and -16.61 ± 0.60 , respectively. From the Gram-positive bacteria, *S. epidermidis* and *B. cereus* were the most hydrophobic with %adhesion of 59.04 ± 1.80 and 18.55 ± 1.00 , respectively, while *S. epidermidis* and *L. acidophilus* had the most negative ζ -potential with -14.11 ± 0.70 and -7.40 ± 0.41 , respectively.

Table 3.2 ζ -potential (mV) and hydrophobicity of bacterial cell surfaces. Hydrophobicity was determined by % of cells that adhere to hexane. Results are taken from a minimum of 3 independent experiments and shown as means \pm standard deviation.

Bacterial species and strain	% adhesion	ζ -potential (mV)
<i>E. coli</i> (MG1655)	0.00 \pm 0.00	-15.20 \pm 0.10
<i>E. coli</i> (BW25113)	0.64 \pm 1.10	-14.61 \pm 0.40
<i>E. coli</i> (JM109)	10.10 \pm 3.70	-16.63 \pm 0.11
<i>E. coli</i> (PHL644)	4.10 \pm 2.00	-16.61 \pm 0.60
<i>E. coli</i> (MC4100)	1.91 \pm 3.30	-15.72 \pm 0.62
<i>S. epidermidis</i>	59.04 \pm 1.80	-14.11 \pm 0.70
<i>B. cereus</i>	18.55 \pm 1.00	-4.50 \pm 0.31
<i>L. acidophilus</i>	8.63 \pm 3.30	-7.40 \pm 0.41

To find out if the O/W emulsion surface properties are different to that of bacteria the O/W emulsions were characterised in terms of their ζ -potential (Table 2.3). Both the coarse and nano-emulsion had slightly negative ζ -potentials with -1.40 \pm 0.32 and -0.61 \pm 0.10, respectively. Since the non-ionic Tween80 is uncharged, the negative ζ -potentials in O/W emulsions were probably due to the presence of impurities.

Table 3.3 ζ -potential (mV) of the coarse and nano-emulsion droplet surfaces. The emulsions were made with sunflower oil (dispersed phase) and M9 minimal growth medium (continuous phase) (ratio of 40:60) stabilised with 8% Tween80. Results are taken from a minimum of 3 independent experiments and shown as means \pm standard deviation.

O/W emulsion	ζ -potential (mV)
Coarse emulsion	-1.40 \pm 0.32
Nano-emulsion	-0.61 \pm 0.10

3.3.3 Effect of oil droplet size on the survival of bacteria

To find out if oil globule size in O/W emulsion affects the survival of bacteria, the viability of bacteria in nano-emulsion and coarse emulsion (M9 minimal growth medium as continuous phase) or M9 minimal growth medium (control) was

measured over time (Figures 3.1-3.7). Also, the membrane integrity of bacterial cells was assessed using flow cytometry staining with PI (Table 3.4).

With all the bacterial strains tested there was no significant difference in log CFU/ml with nano-emulsion compared to coarse emulsion and M9 minimal growth medium (Figures 3.1-3.7). The experiment with *L. acidophilus* was discontinued because the bacteria could not survive at day 2. These results suggest that bacterial survival was not affected by changing the size of the droplets in O/W emulsions.

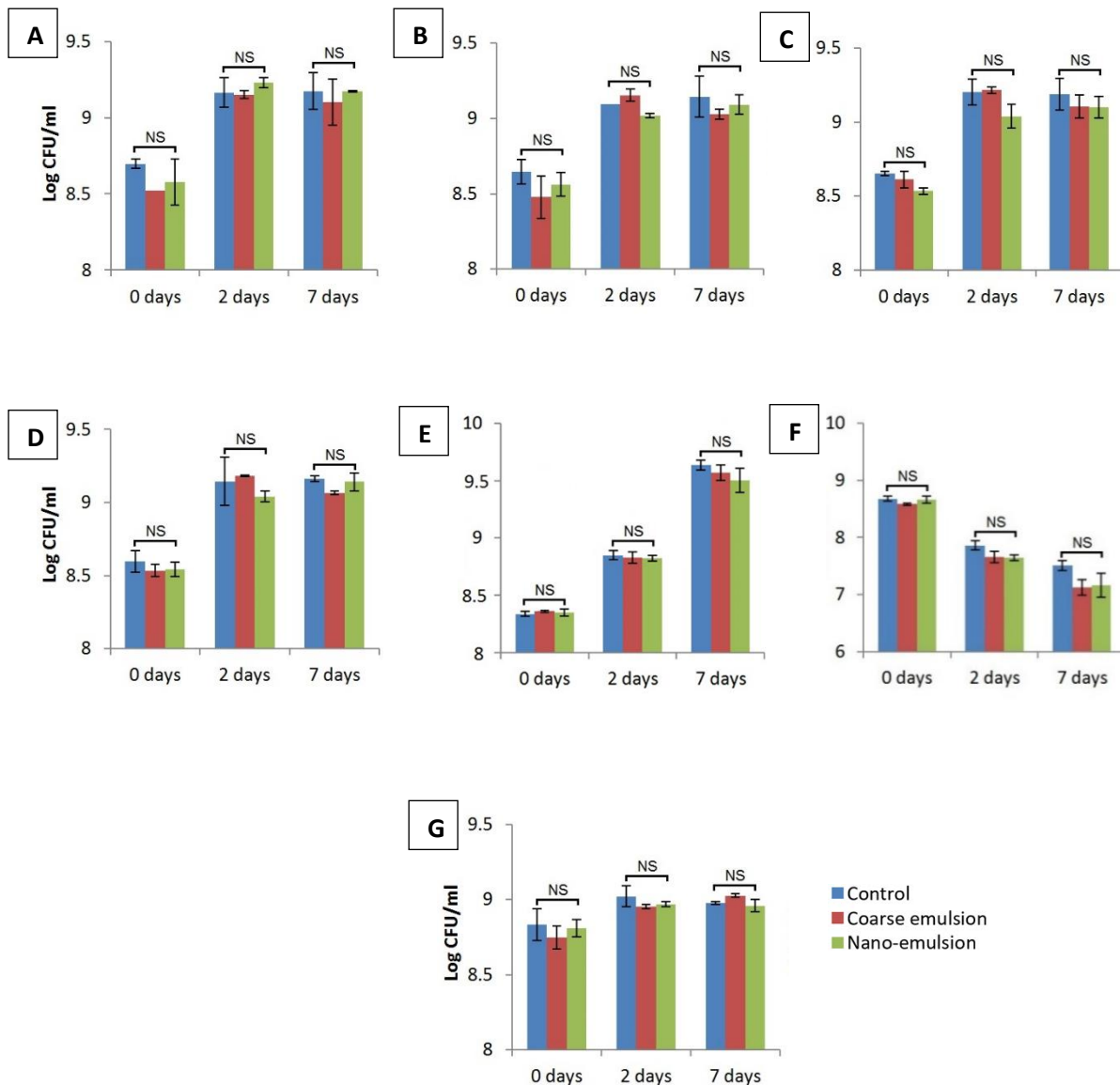


Figure 3.1 Changes in log CFU/ml of *E. coli* (MG1655) (A), *E. coli* (BW2115) (B), *E. coli* (JM109) (C), *E. coli* (MC4100) (D), *E. coli* (PHL644) (E), *B. cereus* (F) and *S. epidermidis* (G) within M9 minimal growth medium (control), nano-emulsion or coarse emulsion at day 0, 2, and 7 incubated at 25°C. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (M9 minimal growth medium). Bars represent mean ± SEM taken from a minimum of 2 independent experiments. The data was analysed with one-way ANOVA.

Flow cytometry results show no significant difference in PI positive cells between all the samples (Table 3.4). Since PI only penetrates cells with compromised membranes the results show that the membrane integrity was not compromised after exposure to nano-emulsion.

Table 3.4 Percentage of PI positive (dead) bacterial cells measured by flow cytometry at 0, 2 and 7-day incubation at 25°C. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (M9 minimal growth medium) in the presence or absence of bacteria. Results are taken from a minimum of 2 independent experiments. Comparisons were made between samples at different time points within each species.

Bacteria	Sample	Day 0	Day 1	Day 7
<i>E. coli</i> (MG1655)	M9 medium	1.00±0.00 ^a	0.30±0.00 ^{abc}	0.15±0.07 ^c
	Coarse emulsion	1.15±0.35 ^{ab}	0.65±0.21 ^{ac}	0.20±0.00 ^c
	Nano-emulsion	0.75±0.21 ^{abc}	0.65±0.21 ^{ac}	0.25±0.07 ^c
<i>E. coli</i> (BW2115)	M9 medium	1.15±0.07 ^d	1.20±0.00 ^d	3.95±0.21 ^a
	Coarse emulsion	0.80±0.14 ^d	0.55±0.07 ^d	5.45±0.63 ^b
	Nano-emulsion	0.60±0.00 ^d	0.60±0.14 ^d	2.30±0.14 ^c
<i>E. coli</i> (JM109)	M9 medium	2.20±0.00 ^a	0.70±0.28 ^c	0.40±0.00 ^c
	Coarse emulsion	1.40±0.14 ^b	1.00±0.14 ^{bc}	0.55±0.07 ^c
	Nano-emulsion	1.30±0.14 ^b	0.45±0.07 ^c	0.40±0.14 ^c
<i>E. coli</i> (MC4100)	M9 medium	3.95±0.35 ^{ab}	5.20±1.27 ^{ab}	8.40±0.28 ^a
	Coarse emulsion	3.55±0.21 ^{ab}	3.20±0.85 ^{ab}	7.95±0.35 ^a
	Nano-emulsion	2.75±0.49 ^{ab}	2.60±0.00 ^b	6.55±3.60 ^{ab}
<i>E. coli</i> (PHL644)	M9 medium	0.30±0.14 ^{acde}	0.40±0.14 ^{ade}	0.30±0.14 ^{acde}
	Coarse emulsion	0.30±0.14 ^{acde}	0.15±0.07 ^e	0.30±0.14 ^{acde}
	Nano-emulsion	0.15±0.07 ^{de}	0.15±0.07 ^e	0.15±0.07 ^{de}
<i>B. cereus</i>	M9 medium	2.35±0.07 ^a	8.10±1.27 ^b	18.70±6.93 ^c
	Coarse emulsion	2.70±0.00 ^a	8.35±1.06 ^b	22.10±2.00 ^{cd}
	Nano-emulsion	2.95±0.35 ^a	6.90±0.42 ^b	30.75±0.63 ^d
<i>S. epidermidis</i>	M9 medium	0.10±0.00 ^a	0.10±0.00 ^a	0.00±0.00 ^c
	Coarse emulsion	0.05±0.07 ^b	0.10±0.00 ^a	0.00±0.00 ^c
	Nano-emulsion	0.10±0.00 ^a	0.10±0.00 ^a	0.00±0.00 ^c

The data was analysed with one-way ANOVA

a,b,c,d,e means ± standard deviation with different letters are significantly different

3.3.4 Effect of oil droplet size on the growth of bacteria

To find out if oil droplet size in O/W emulsion affects the growth of bacteria, the viability of bacteria in nano-emulsion and coarse emulsion (broth as continuous phase) or broth (control) was measured over time (Fig 3.2A-H).

With all the bacterial strains tested there was no significant difference of increase in log CFU/ml between nano-emulsion, coarse emulsion or broth (Fig 3.2A-H). These

results suggests that bacterial growth is not affected by changing the size of the droplets in O/W emulsions.

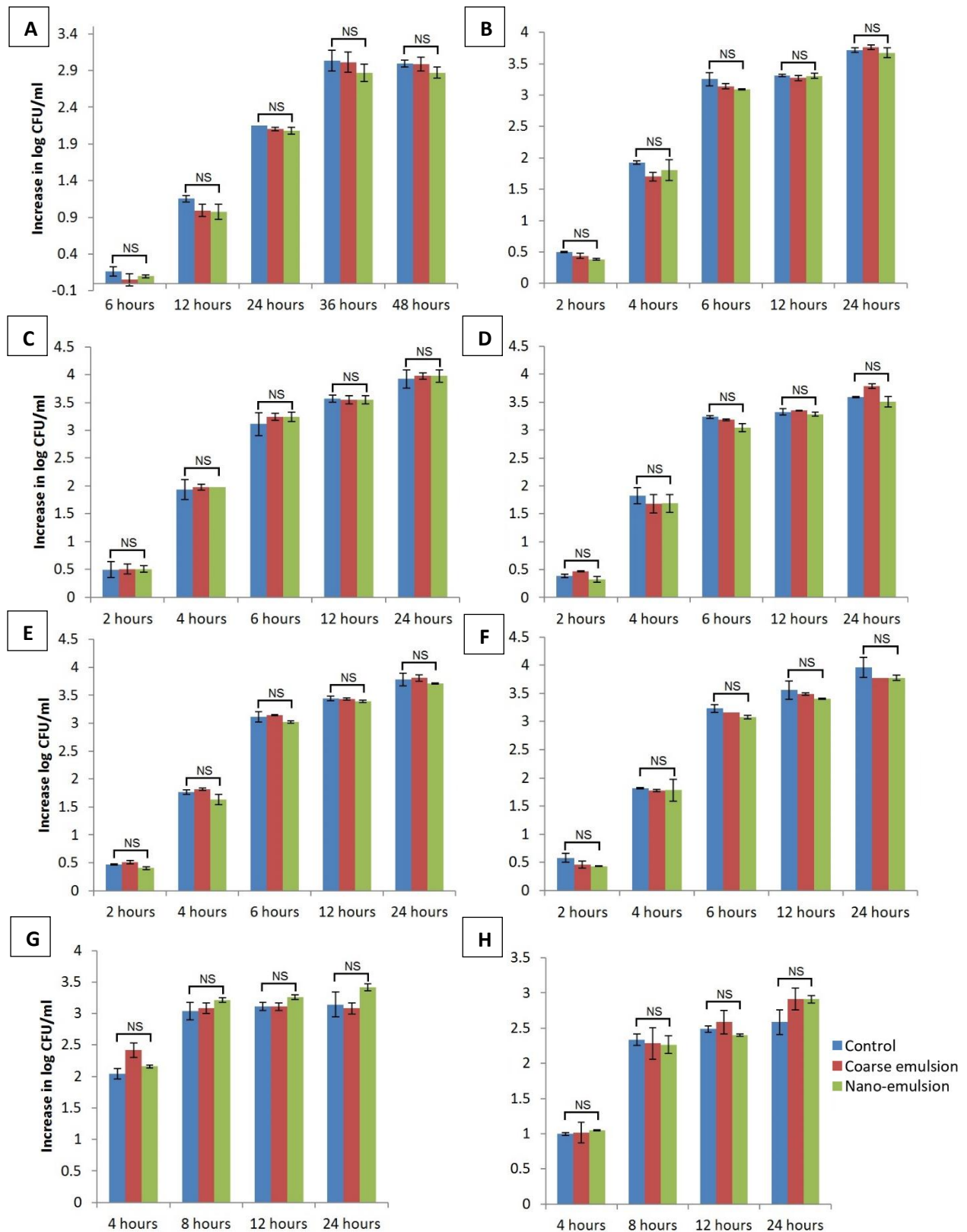


Figure 3.2 Changes in log CFU/ml of *L. acidophilus* (A), *E. coli* (MG1655) (B), *E. coli* (BW2115) (C), *E. coli* (JM109) (D), *E. coli* (MC4100) (E), *E. coli* (PHL644) (F, G, H)

(F), *B. cereus* (G) and *S. epidermidis* (H) within broth (control), nano-emulsion or coarse emulsion over 24 or 48 hours relative to hour 0 incubation at 30°C. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (tryptic soy broth). Bars represent mean \pm SEM taken from a minimum of 2 independent experiments. The data was analysed with one-way ANOVA.

3.3.5 Microscopic observation

The spatial distribution and localisation of the bacterial cells within the O/W emulsions was observed using fluorescence microscopy during survival (Fig 3.3-3.9) and growth (3.10-3.17).

3.3.5.1 Microscopic observation during survival study

In all samples, microscopic observation showed bacteria being present in the continuous phase (M9 minimal growth medium) as planktonic cells with no attachment to the oil globules (Figures 3.3-3.8). These results suggest that the structure of O/W emulsion has no effect on the behaviour of the bacterial cells.

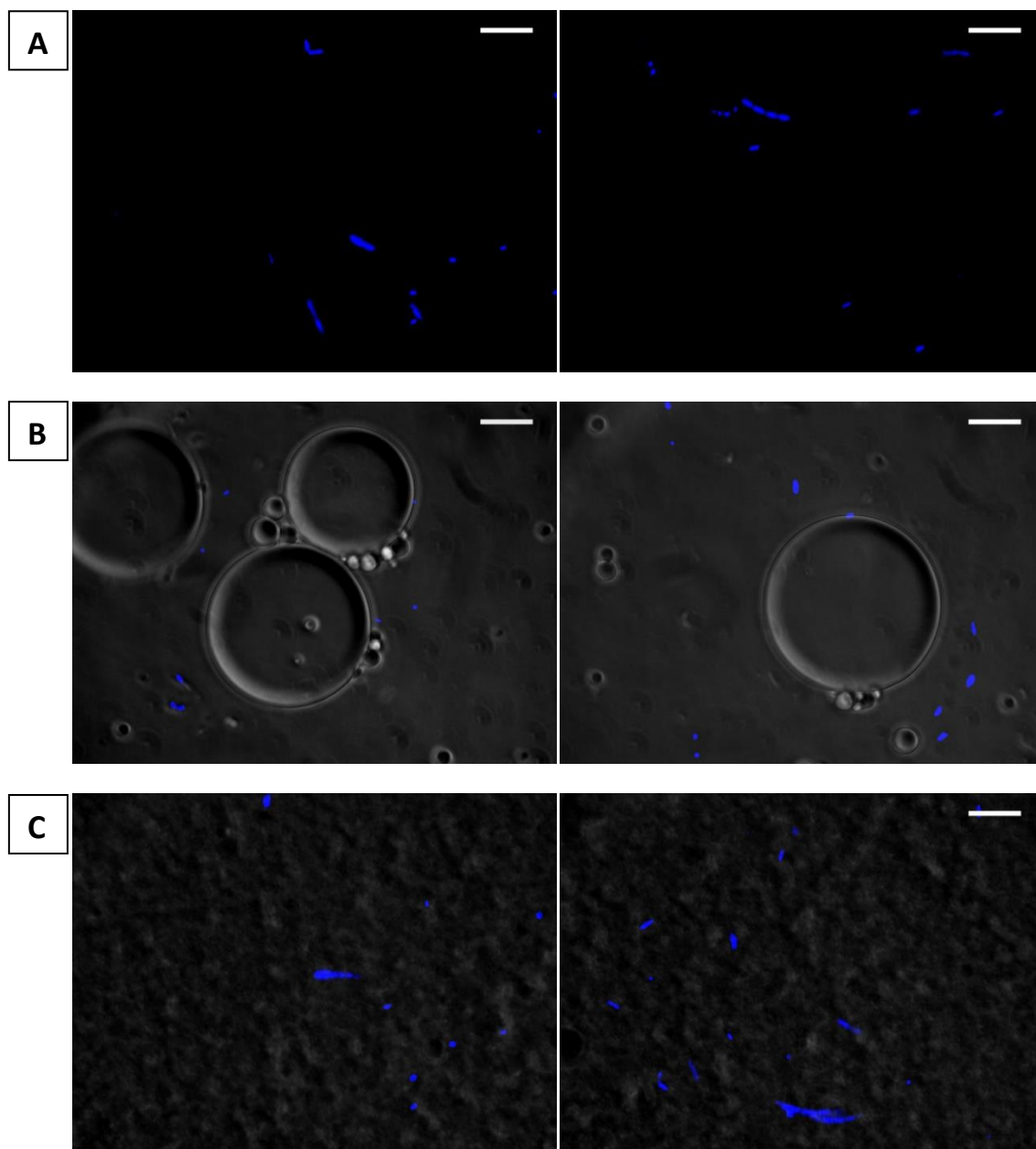


Figure 3.3 Photomicrographs composed from the optical and fluorescence images of *B. cereus* within M9 minimal growth medium (control) (A), coarse emulsion (B) and nano-emulsion (C) at 0 (left) and 7 (right) days. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (M9 media). Scale bar: 10 μ m.

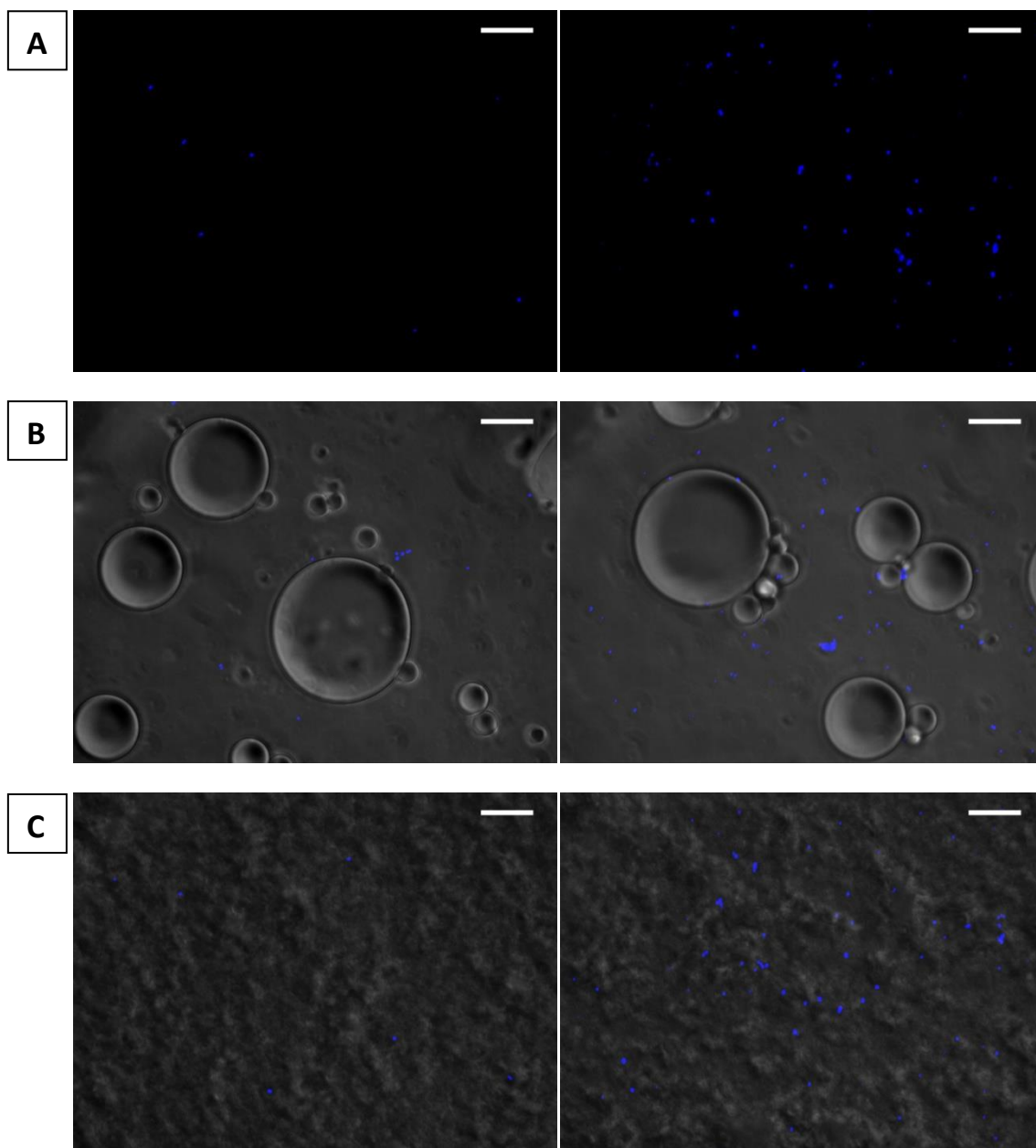


Figure 3.4 Photomicrographs composed from the optical and fluorescence images of *S. epidermidis* within M9 minimal growth medium (control) (A), coarse emulsion (B) and nano-emulsion (C) at 0 (left) and 7 (right) days. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (M9 media). Scale bar: 10 μ m.

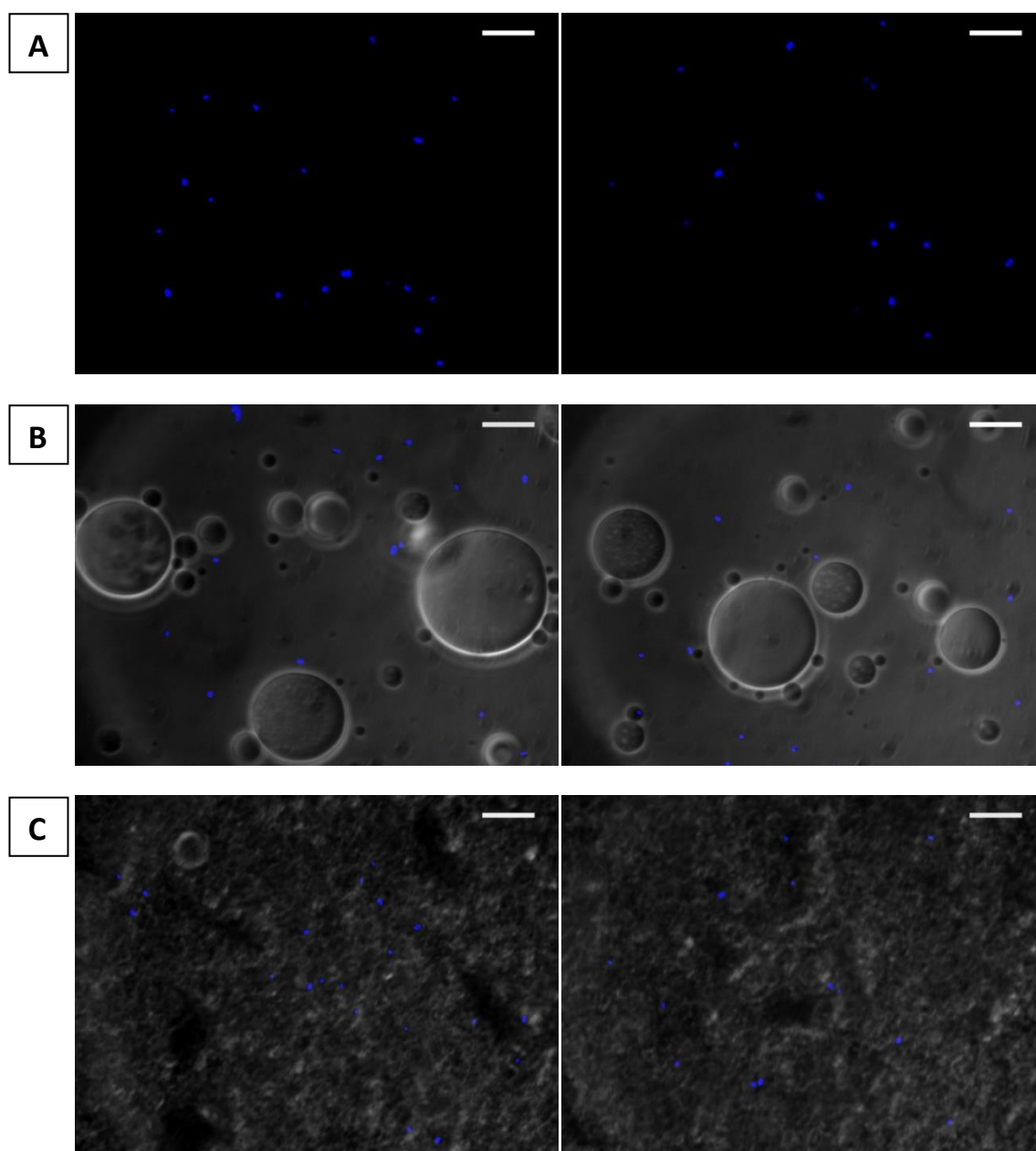


Figure 3.5 Photomicrographs composed from the optical and fluorescence images of *E. coli* (MG1655) within M9 minimal growth medium (control) (A), coarse emulsion (B) and nano-emulsion (C) at 0 (left) and 7 (right) days. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (M9 media). Scale bar: 10 μ m.

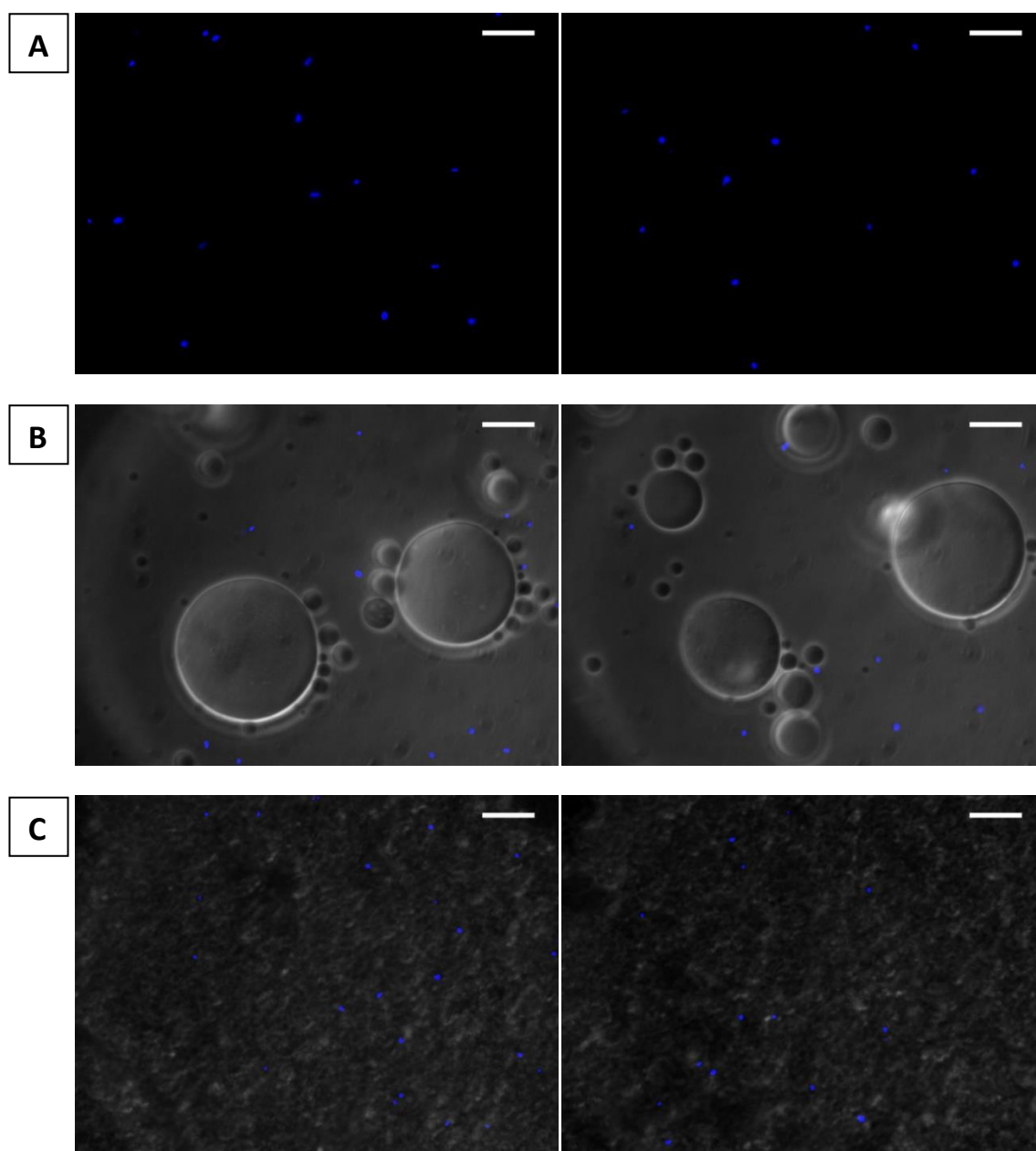


Figure 3.6 Photomicrographs composed from the optical and fluorescence images of *E. coli* (BW2115) within M9 minimal growth medium (control) (A), coarse emulsion (B) and nano-emulsion (C) at 0 (left) and 7 (right) days. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (M9 media). Scale bar: 10 μ m.

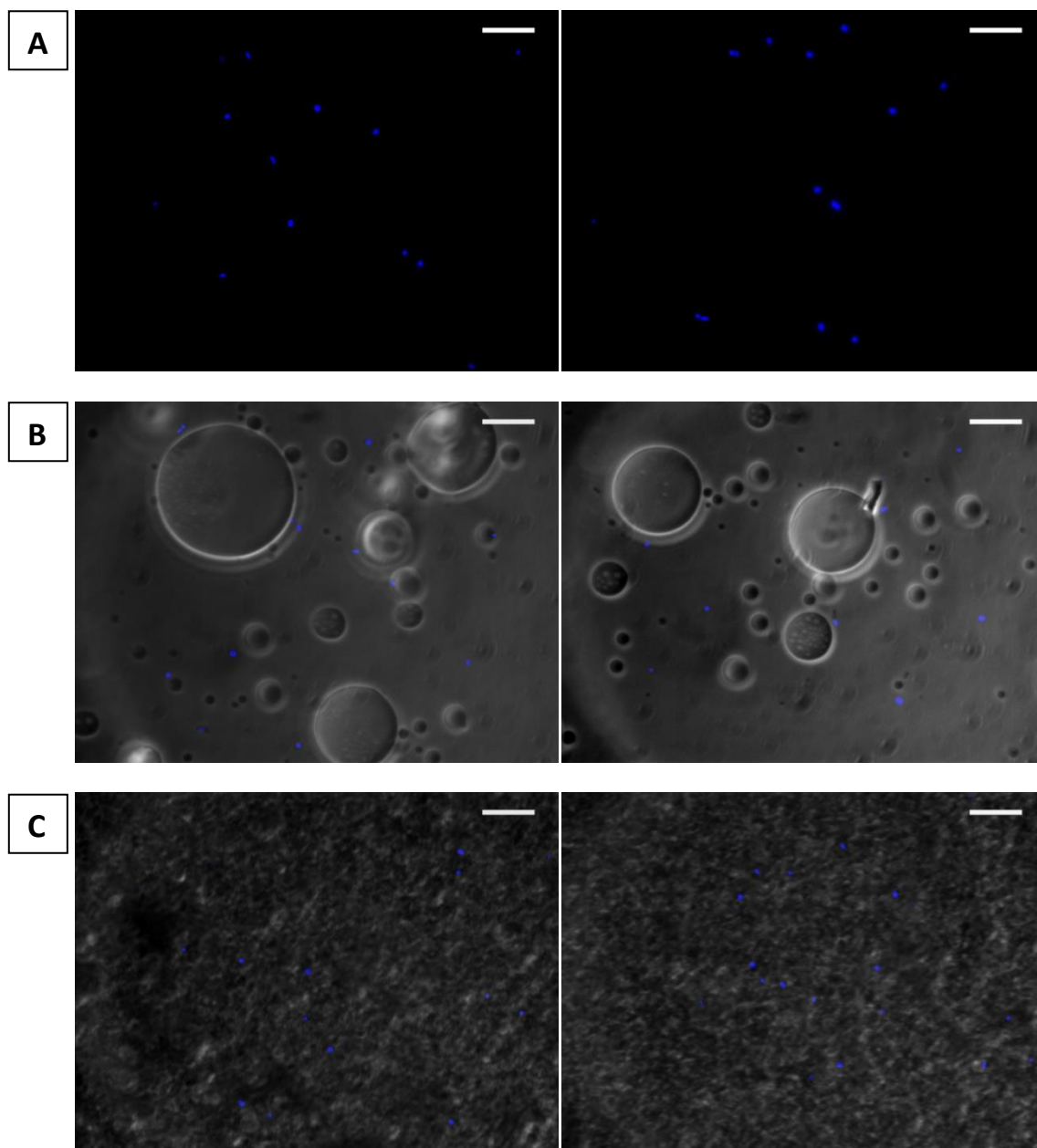


Figure 3.7 Photomicrographs composed from the optical and fluorescence images of *E. coli* (JM109) within M9 minimal growth medium (control) (A), coarse emulsion (B) and nano-emulsion (C) at 0 (left) and 7 (right) days. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (M9 media). Scale bar: 10 μ m.

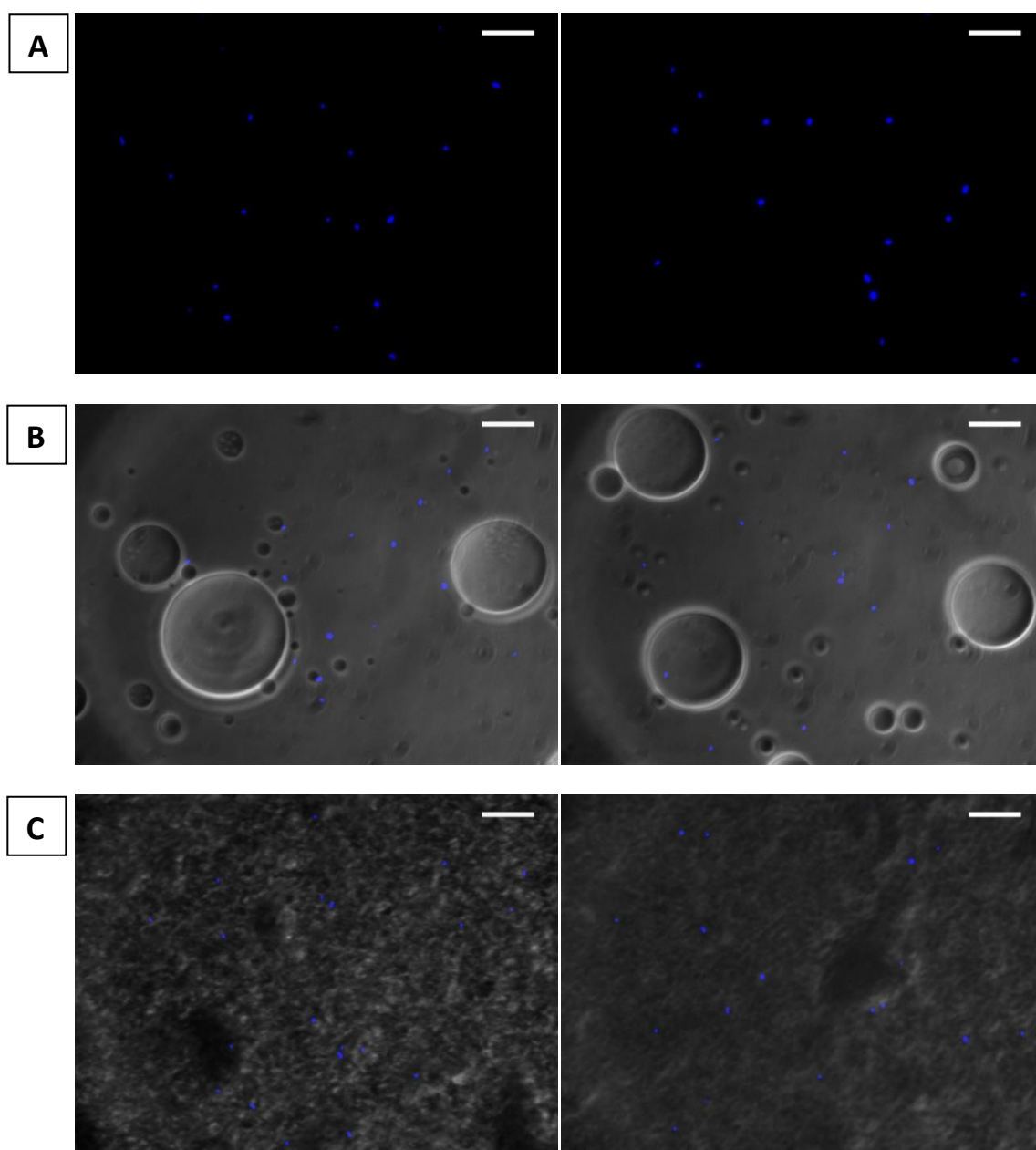


Figure 3.8 Photomicrographs composed from the optical and fluorescence images of *E. coli* (MC4100) within M9 minimal growth medium (control) (A), coarse emulsion (B) and nano-emulsion (C) at 0 (left) and 7 (right) days. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (M9 media). Scale bar: 10μm.

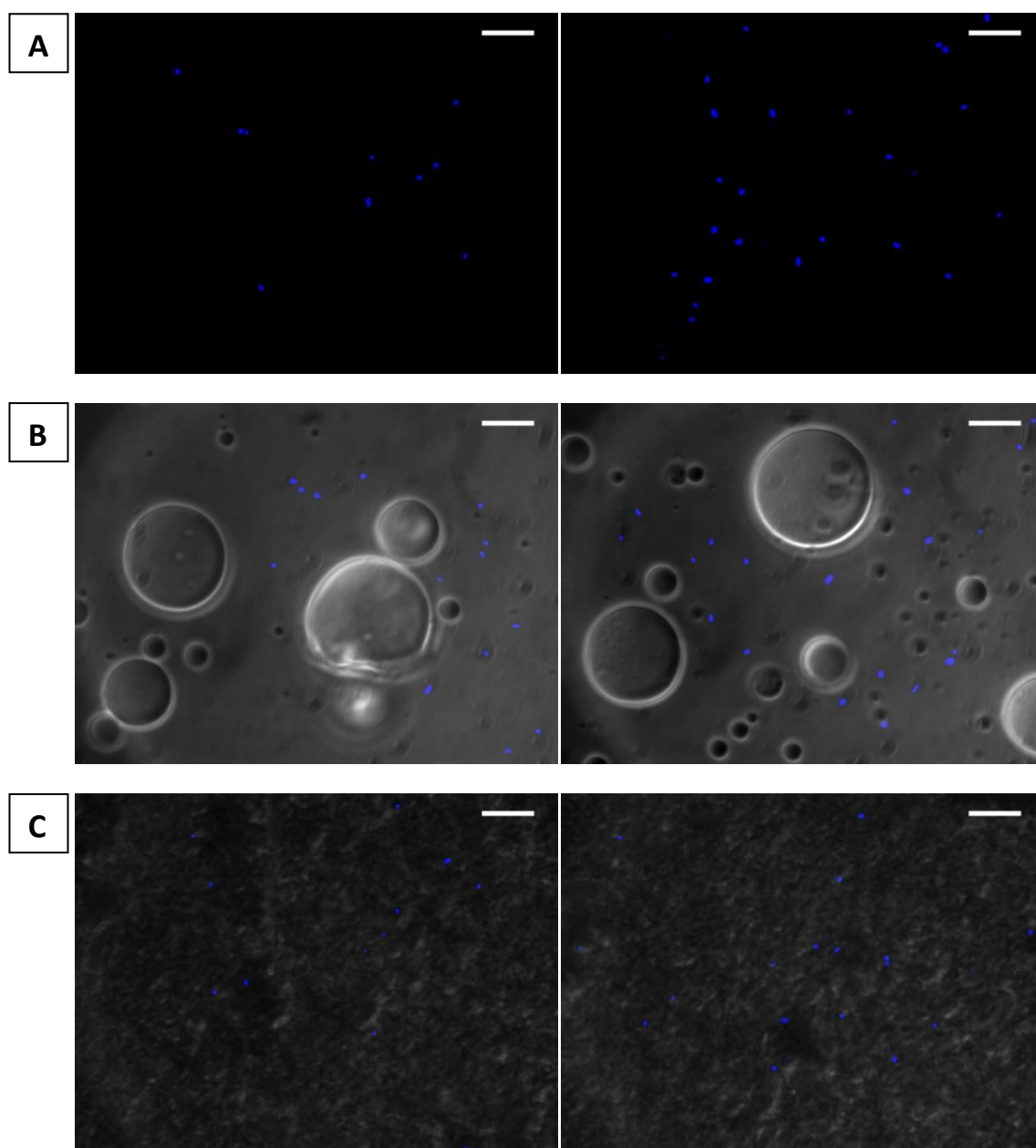


Figure 3.9 Photomicrographs composed from the optical and fluorescence images of *E. coli* (PHL644) within M9 minimal growth medium (control) (A), coarse emulsion (B) and nano-emulsion (C) at 0 (left) and 7 (right) days. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (M9 media). Scale bar: 10 μ m.

2.3.5.2 Microscopic observation during growth study

In all samples, microscopic observation showed bacteria being present in the continuous phase (broth) as planktonic cells with no attachment to the oil globules (Figures 3.10-3.17). These results suggest that the structure of O/W emulsion has no effect on the behaviour of the bacterial cells during growth.

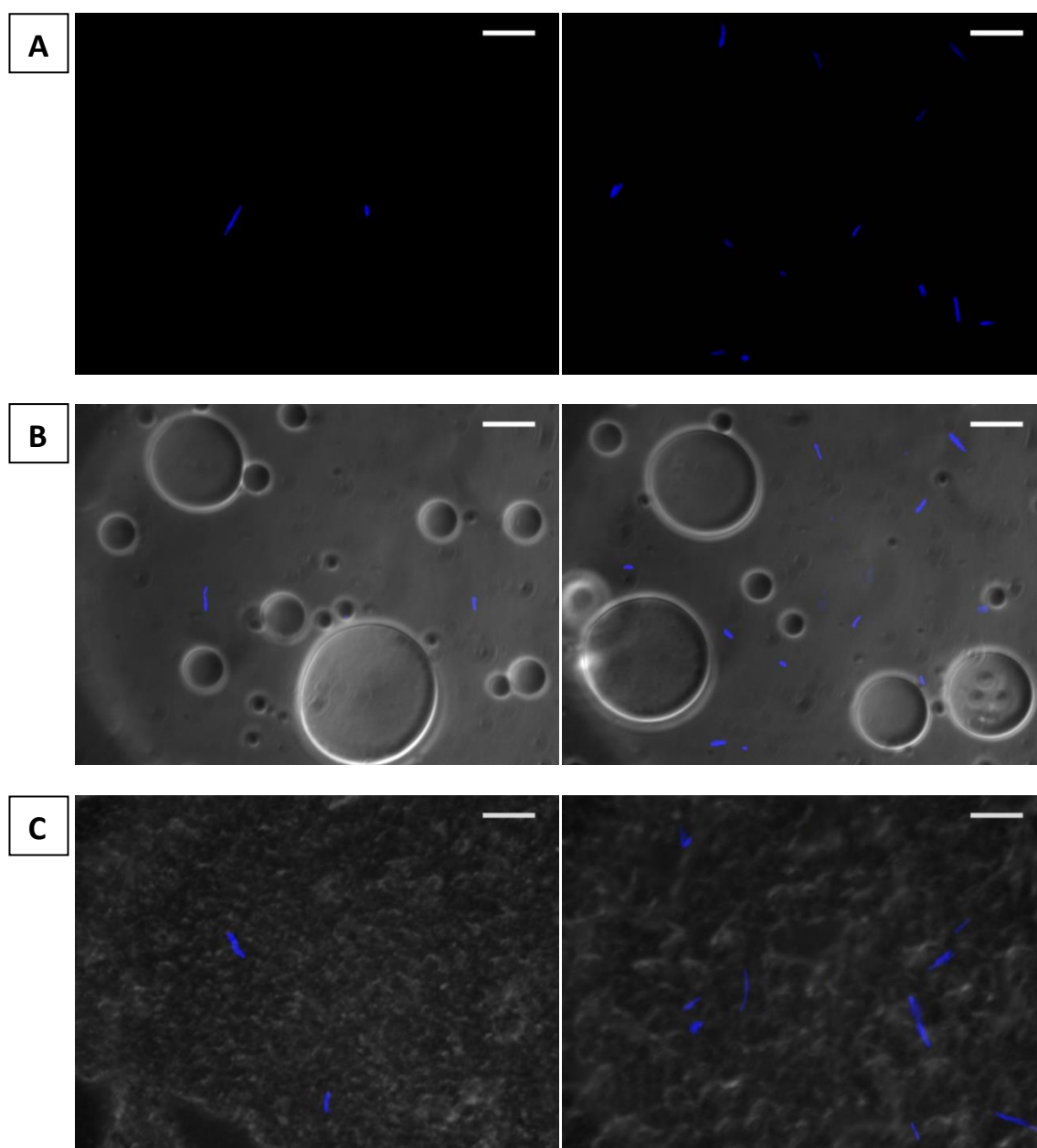


Figure 3.10 Photomicrographs composed from the optical and fluorescence images of *L. acidophilus* within broth (control) (A), coarse emulsion (B) and nano-emulsion (C) at 0 (left) and 48 (right) hours. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (M.R.S broth). Scale bar: 10 μ m.

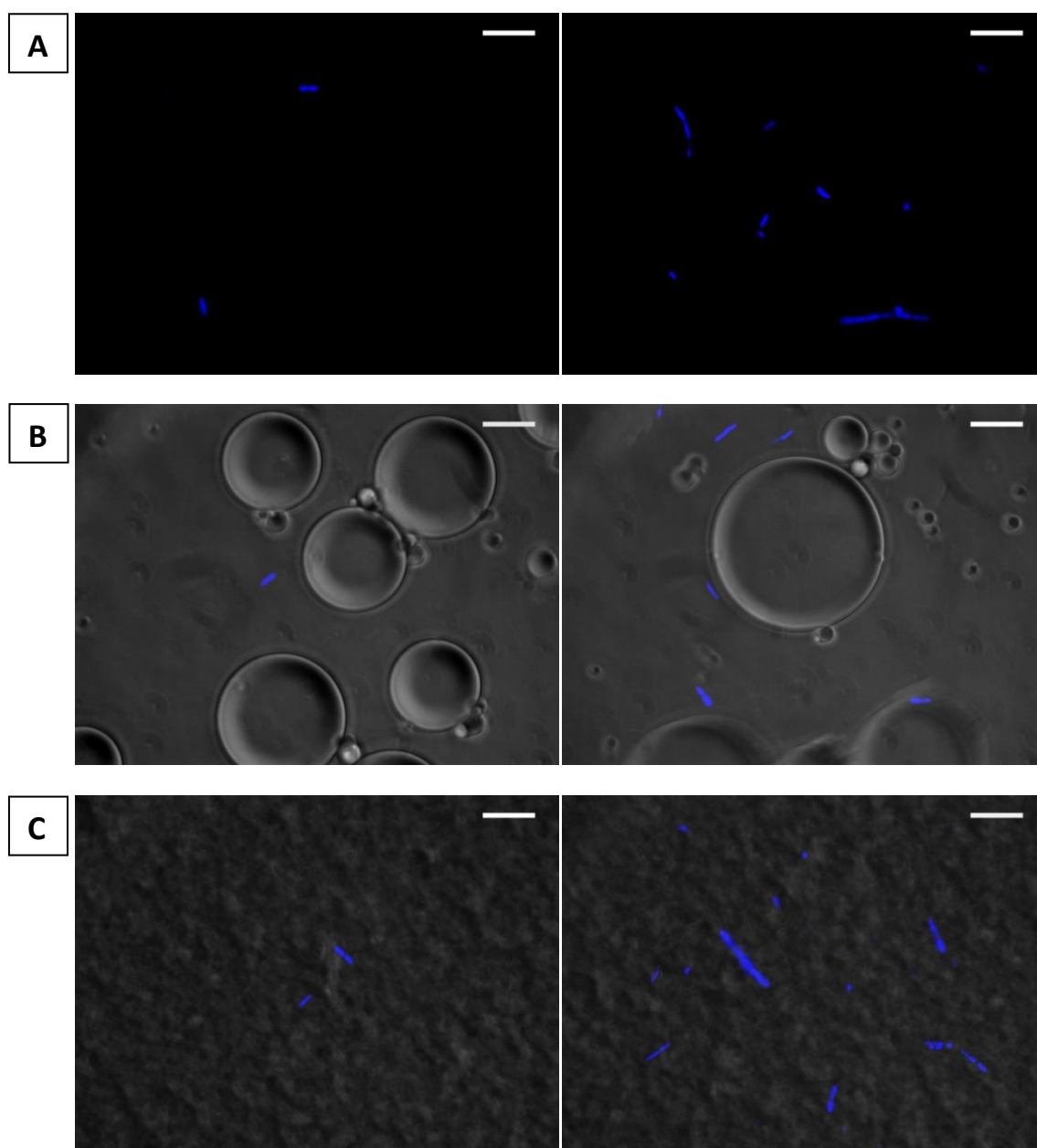


Figure 3.11 Photomicrographs composed from the optical and fluorescence images of *B. cereus* within broth (control) (A), coarse emulsion (B) and nano-emulsion (C) at 0 (left) and 24 (right) hours. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (tryptic soy broth). Scale bar: 10 μ m.

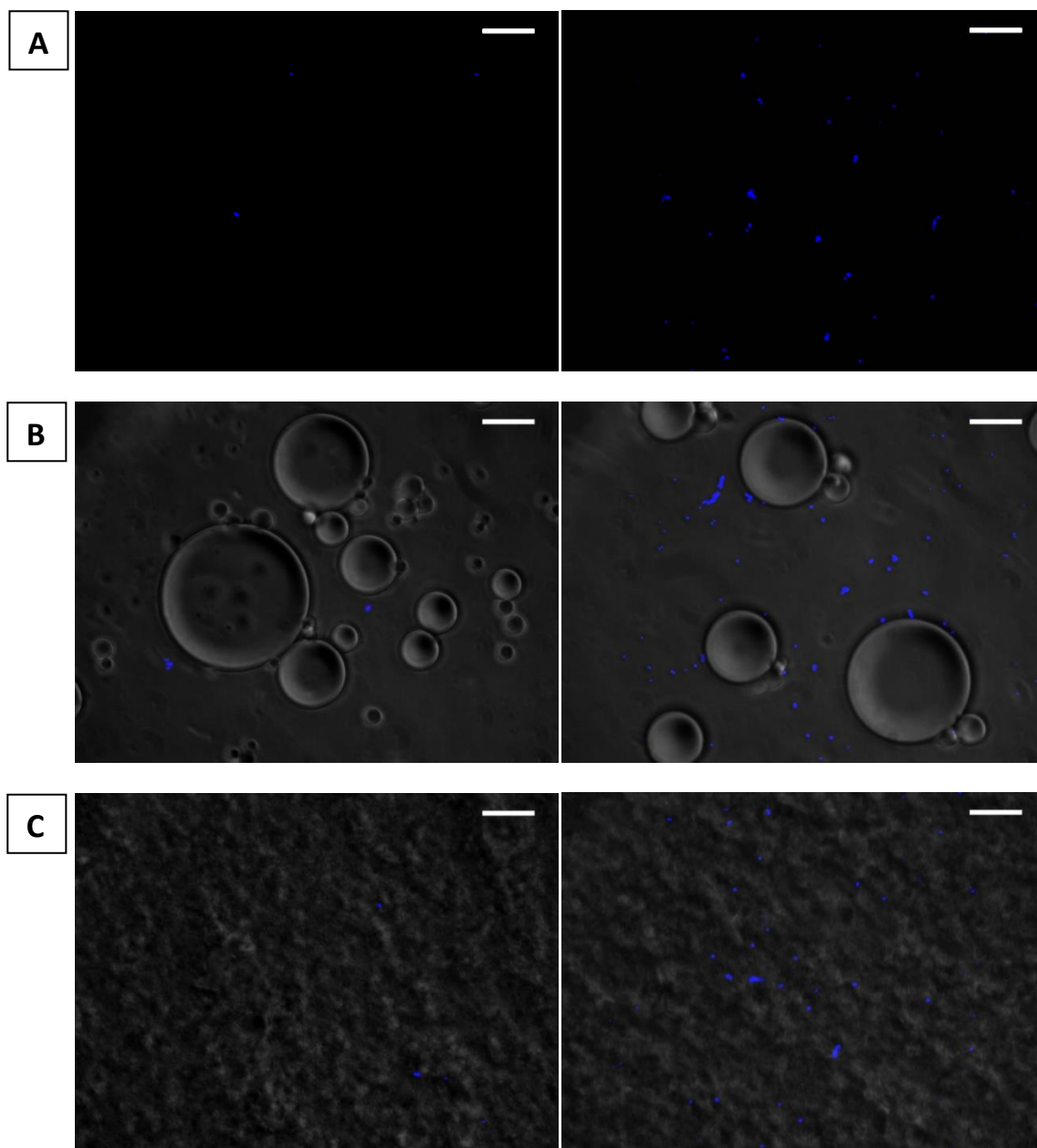


Figure 3.12 Photomicrographs composed from the optical and fluorescence images of *S. epidermidis* within broth (control) (A), coarse emulsion (B) and nano-emulsion (C) at 0 (left) and 24 (right) hours. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (tryptic soy broth). Scale bar: 10 μ m.

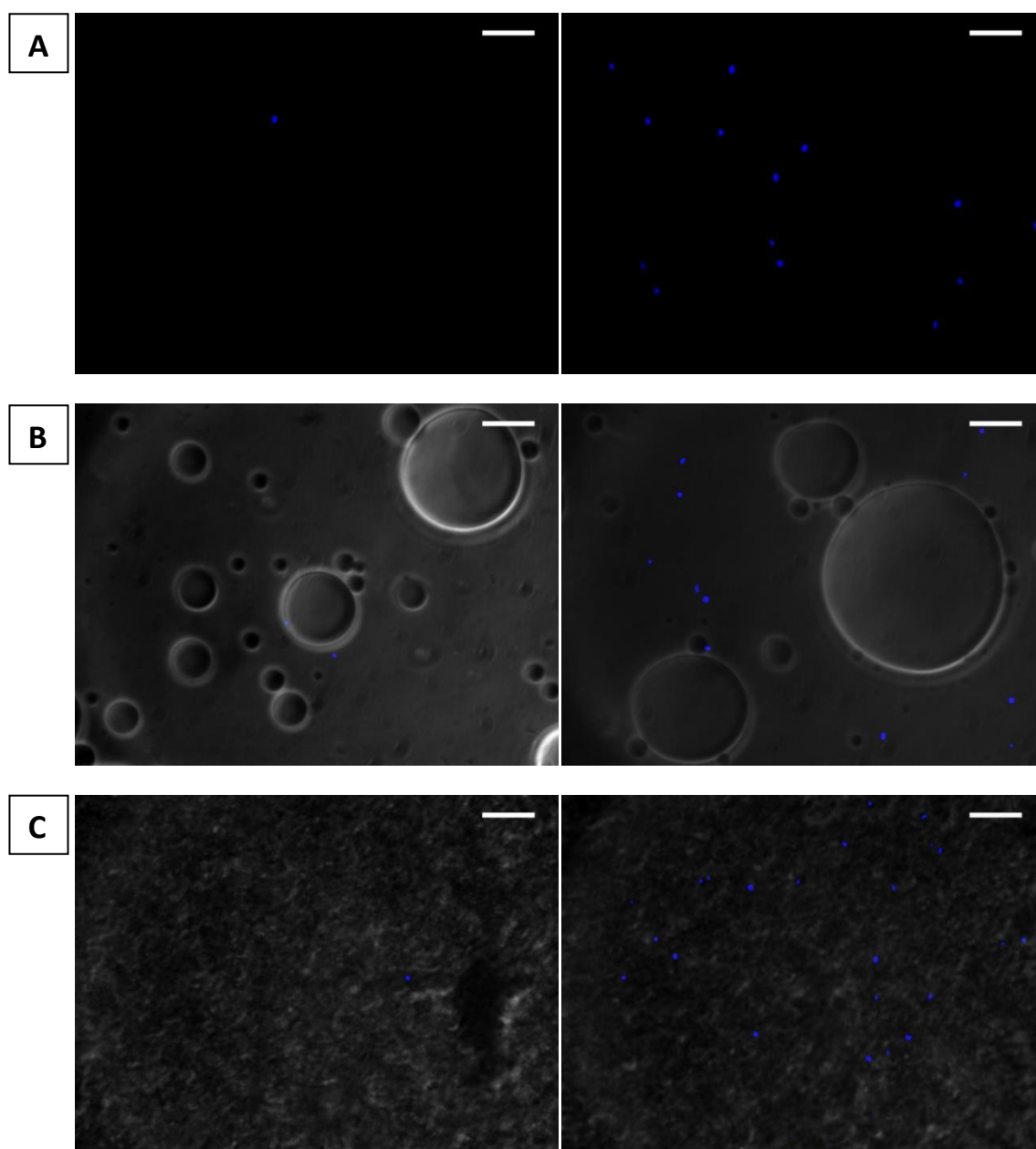


Figure 3.13 Photomicrographs composed from the optical and fluorescence images of *E. coli* (MG1655) within broth (control) (A), coarse emulsion (B) and nano-emulsion (C) at 0 (left) and 24 (right) hours. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (tryptic soy broth). Scale bar: 10 μ m.

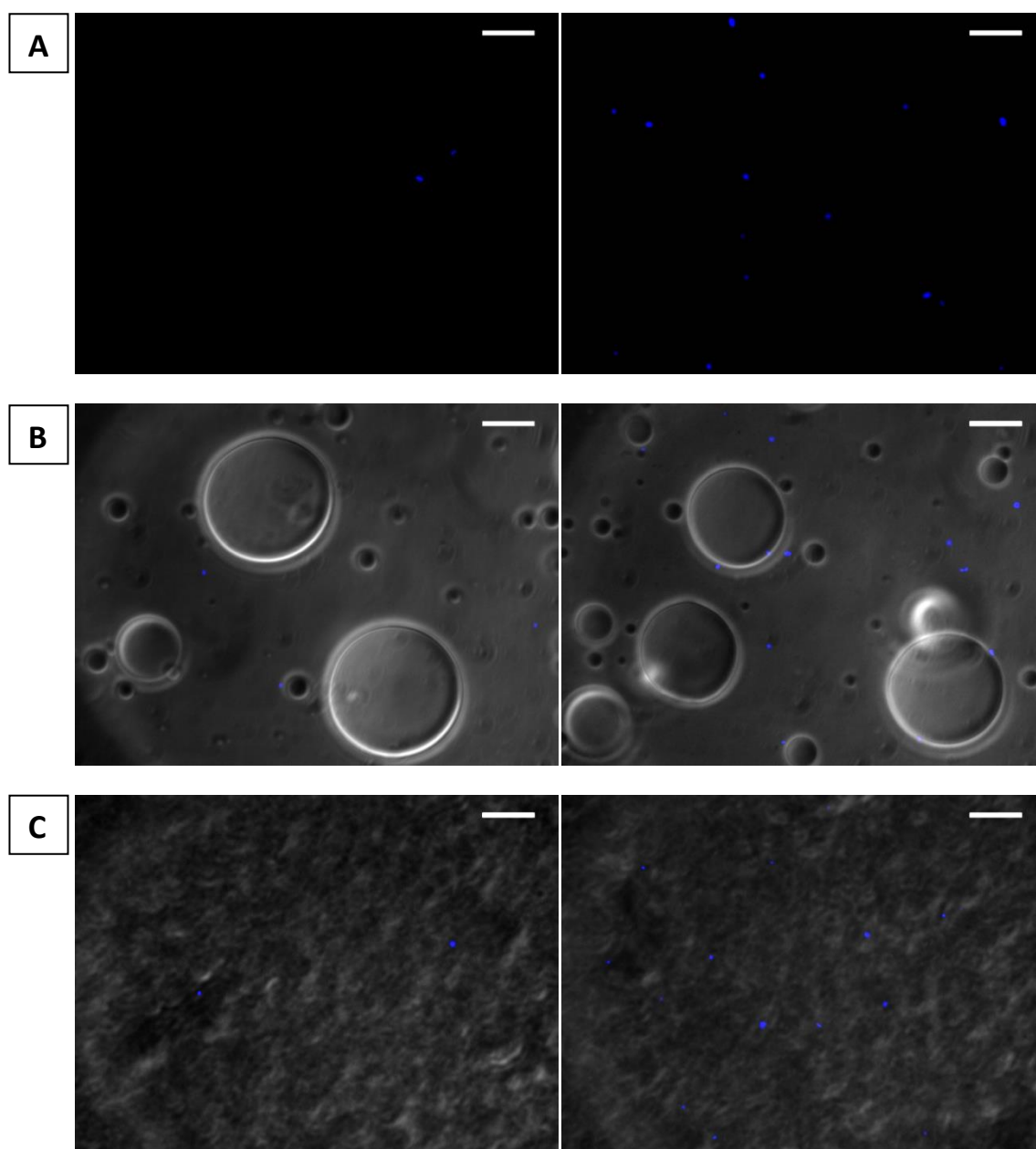


Figure 3.14 Photomicrographs composed from the optical and fluorescence images of *E. coli* (BW2115) within broth (control) (A), coarse emulsion (B) and nano-emulsion (C) at 0 (left) and 24 (right) hours. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (tryptic soy broth). Scale bar: 10 μ m.

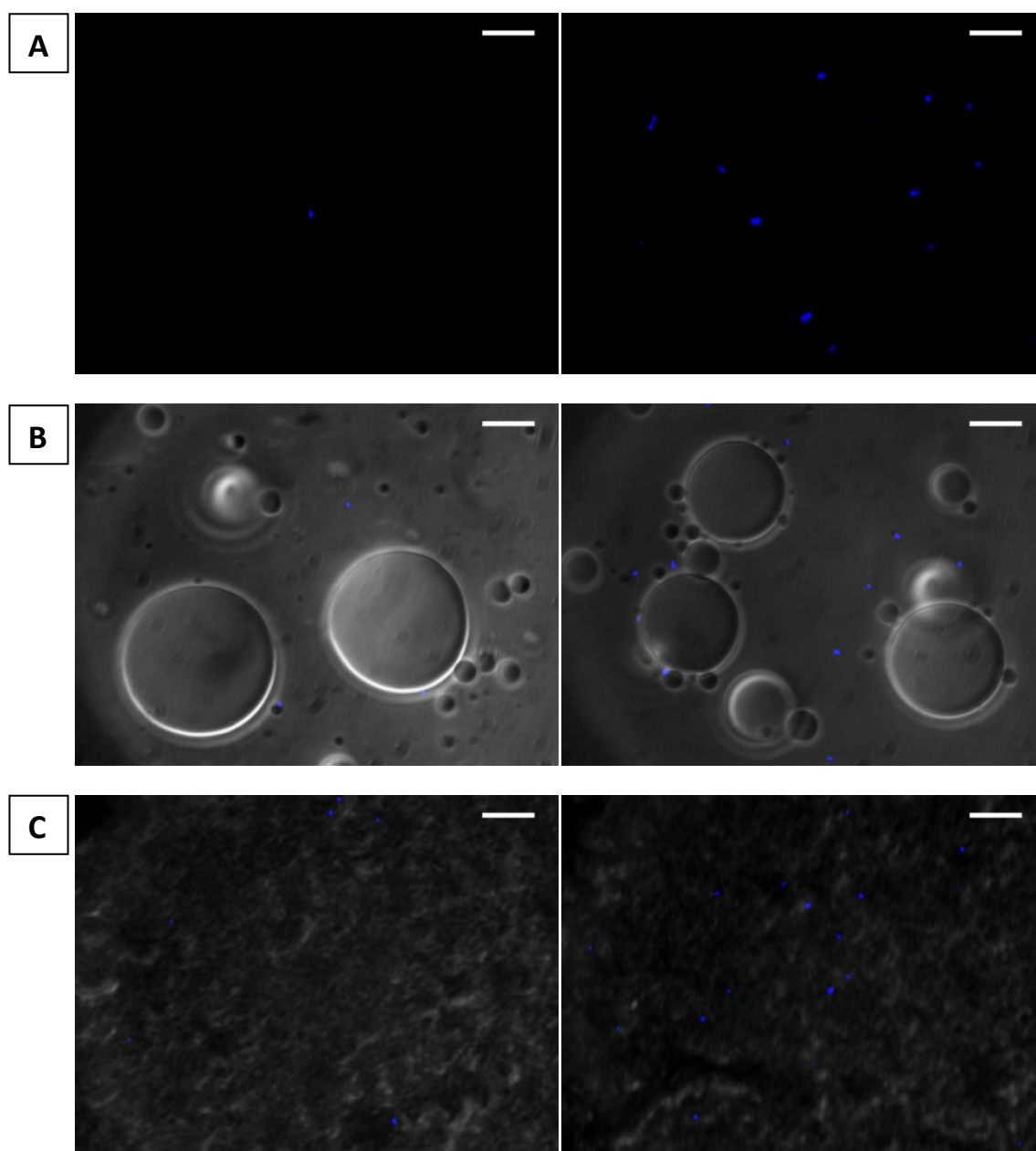


Figure 3.15 Photomicrographs composed from the optical and fluorescence images of *E. coli* (JM109) within broth (control) (A), coarse emulsion (B) and nano-emulsion (C) at 0 (left) and 24 (right) hours. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (tryptic soy broth). Scale bar: 10μm.

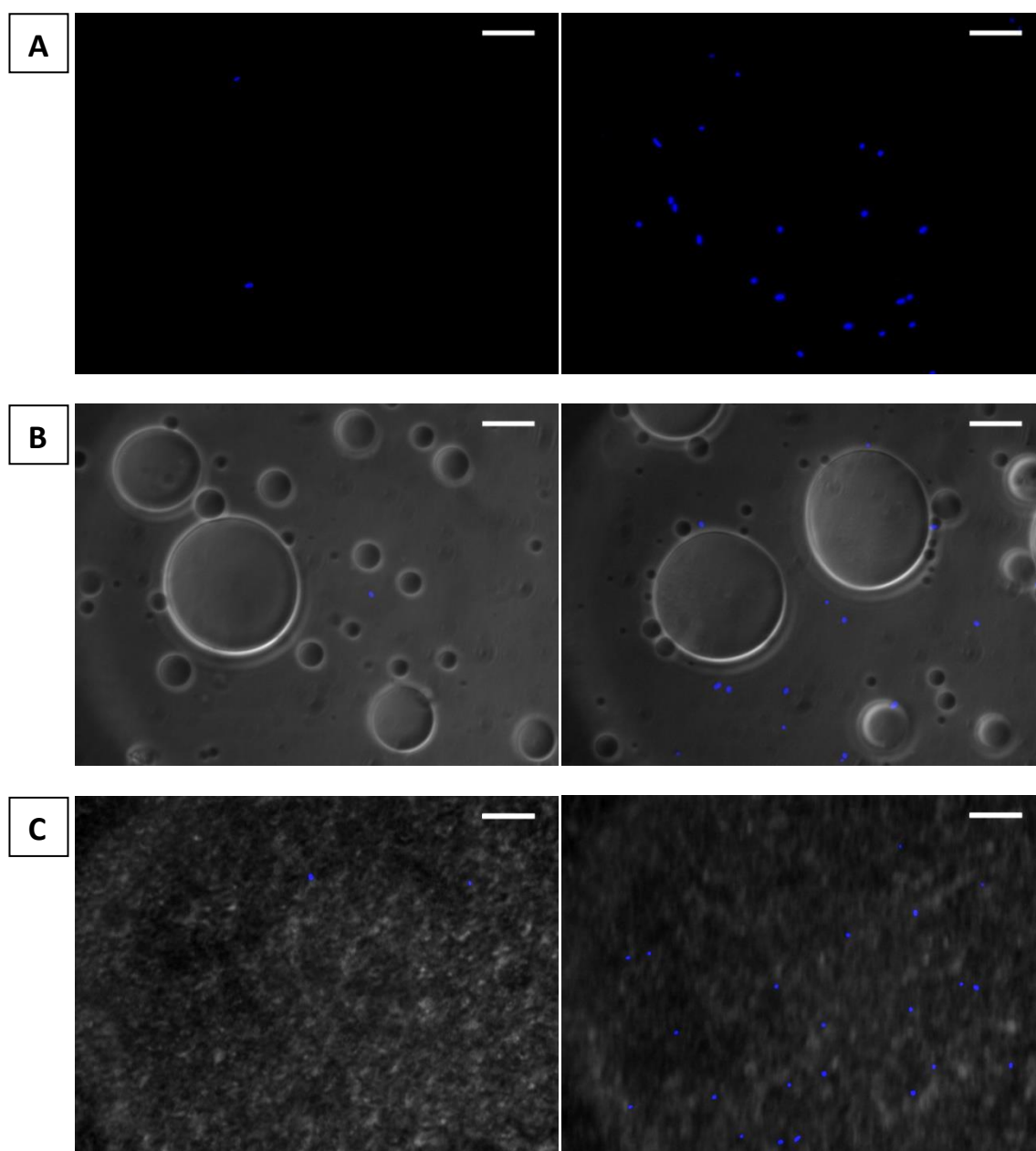


Figure 3.16 Photomicrographs composed from the optical and fluorescence images of *E. coli* (MC4100) within broth (control) (A), coarse emulsion (B) and nano-emulsion (C) at 0 (left) and 24 (right) hours. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (tryptic soy broth). Scale bar: 10 μ m.

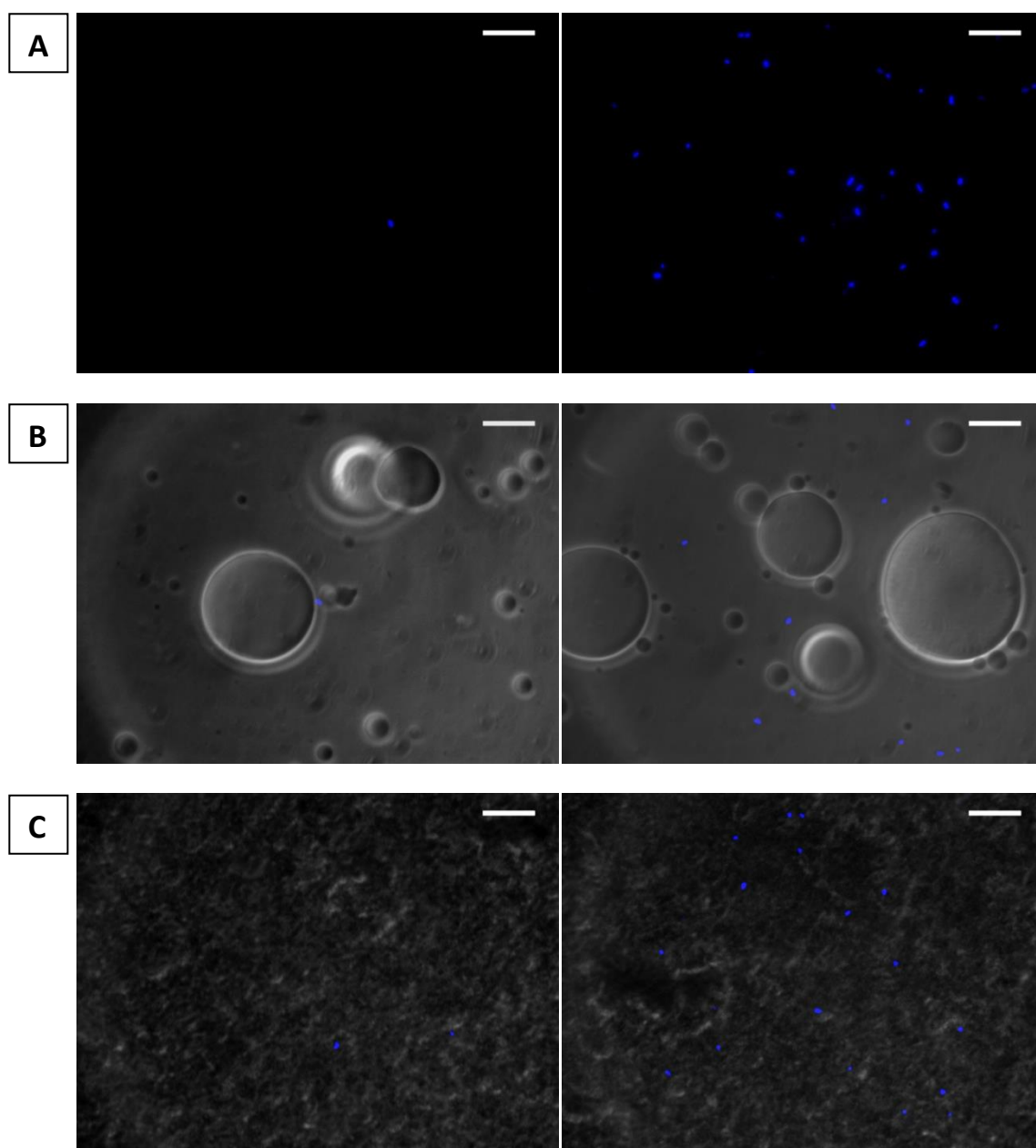


Figure 3.17 Photomicrographs composed from the optical and fluorescence images of *E. coli* (PHL644) within broth (control) (A), coarse emulsion (B) and nano-emulsion (C) at 0 (left) and 24 (right) hours. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (tryptic soy broth). Scale bar: 10 μ m.

3.3.6 Activity of antimicrobial caprylic acid in O/W emulsion of different droplet size and concentration of Tween80

The effect of reducing the size of the oil droplets on the antimicrobial activity of O/W emulsions containing CA was investigated (Fig 3.18). In non-homogenised sample, the viability of *S. epidermidis* significantly ($P < 0.05$) decreased with increasing concentrations of CA. After 1 hour, all non-homogenised samples showed high viability (> 8 -log CFU/ml) while after 8 hours non-homogenised sample containing 1% CA showed < 2 -log CFU/ml. However, the viability of bacteria in non-homogenised sample containing 0.5% CA was significantly ($P < 0.05$) lower (~ 4 -log CFU/ml) compared to no CA. After 24 hours both non-homogenised samples containing 1 and 0.5% CA showed < 2 -log CFU/ml while non-homogenised sample containing no CA maintained high viability (> 8 -log CFU/ml). The viability of *E. coli* in non-homogenised sample containing 1% CA and no CA showed a similar trend as with *S. epidermidis*. Although the viability of *E. coli* significantly ($P < 0.05$) decreased in non-homogenised sample containing 0.5% CA over time, the viability was ~ 6.5 -log CFU/ml after 24 hours. The O/W emulsions without CA showed no antimicrobial activity against *E. coli* and *S. epidermidis*. The antimicrobial activity of CA was significantly ($P < 0.05$) increased in nano-emulsion and coarse emulsion compared to non-homogenised sample after 8 and 24 hours. For both bacterial species, the antimicrobial activity of CA followed a similar trend in nano-emulsion compared to coarse emulsion and there no significant difference in viability at 24 hours. These results suggest that CA possess higher antimicrobial activity in O/W emulsion compared to non-homogenised sample but was not different between nano-emulsion and coarse emulsion.

The effect of altering the concentration of Tween80 (1% and 8%) on the antimicrobial activity of O/W emulsions containing CA was investigated. The antimicrobial activity of CA was significantly ($P<0.05$) higher against *E. coli* (MG1655) and *S. epidermidis* in nano-emulsion and coarse emulsion stabilised with 1% compared to 8% Tween80. The viability of both bacteria was $>6\text{-log CFU/ml}$ after 24 hours with 8% Tween80 while the viability was $<2\text{-log CFU/ml}$ after 8 hours with 1% Tween80. These results suggest that higher concentrations of Tween80 reduces the antimicrobial effects of CA against the bacteria.

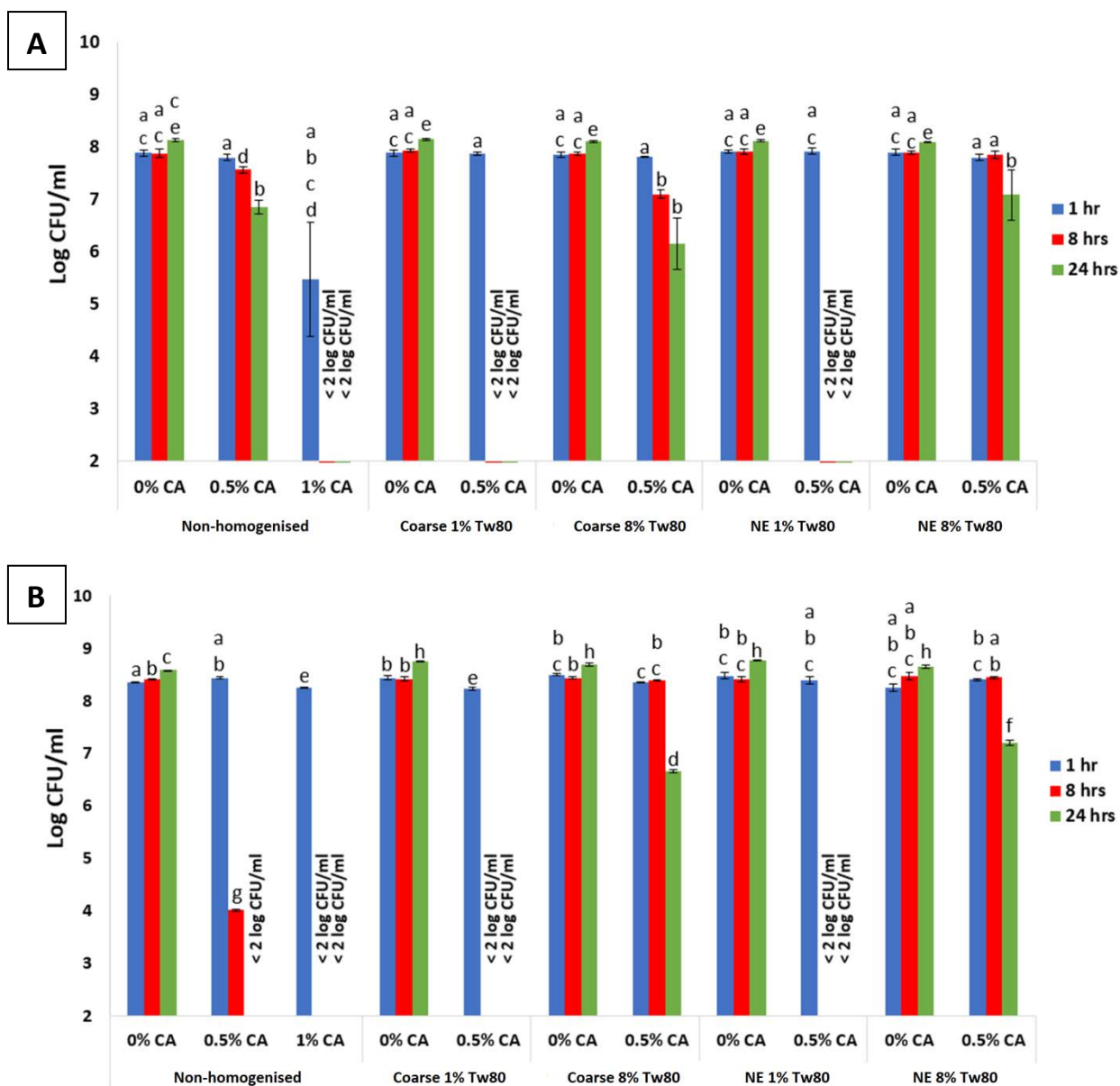


Figure 3.18 Log CFU/ml of *E. coli* (MG1655) (A) and *S. epidermidis* (B) in non-homogenised sample (control), nano-emulsion (NE), or coarse emulsion after 1, 8 and 24 hours incubated at 25°C. The O/W emulsions were prepared with 20% oil phase containing no or 0.5% CA and stabilised with 1 or 8% Tween80 in M9 minimal growth medium (continuous phase). The non-homogenised sample was prepared from 20% oil phase containing 0, 0.5 and 1% CA and M9 minimal growth medium. Bars represent mean \pm SEM taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different ($P < 0.05$). The data was analysed with one-way ANOVA. Abbreviations: NE, nano-emulsion; CA, caprylic acid.

3.3.7 Effect of bacteria on stability of O/W emulsions

To understand how the presence of bacteria affected the stability of the oil globules within the different formulations of O/W emulsions, the oil globule size $[D(4, 3)]$ and creaming thickness (data not shown) with or without bacteria in the continuous phase for study with CA (Fig 3.19) or without CA (Table. 3.4) was measured over time.

There was no significant difference in the $D(4, 3)$ or creaming stability of nano-emulsion or coarse emulsions with compared to without bacteria in the continuous phase (Fig 3.19 and Table 3.4). These results suggest that the presence of bacteria does not affect the stability of the O/W emulsions.

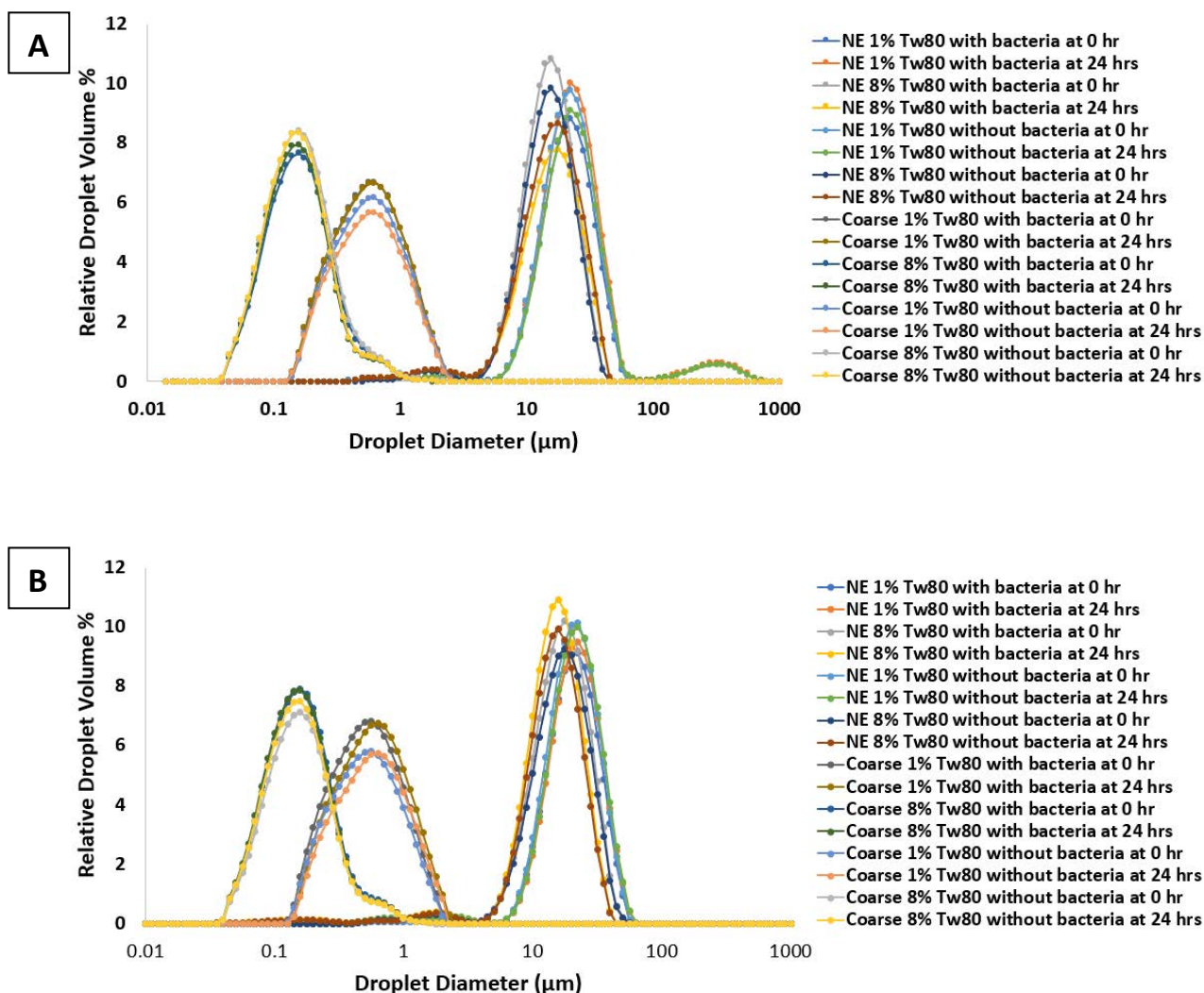


Figure 3.19 The mean diameter size (μm) of the oil droplets by light scattering [D (4, 3)] of *E. coli* (MG1655) (A) and *S. epidermidis* (B) nano-emulsion (NE) or coarse emulsion at 0 and 24 hours incubated at 25°C. The O/W emulsions were prepared with 20% oil phase containing 0.5% CA and stabilised with 1% or 8% Tween80 in M9 minimal growth medium (continuous phase) with or without bacteria. Abbreviations: NE, nano-emulsion; CA, caprylic acid.

Table 3.5 Mean oil globule diameter size (μm) of coarse emulsions and nano-emulsions measured by light scattering [D (4, 3)] at day 0 and after 7-day incubation. The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the presence or absence of bacteria. Results are taken from a minimum of 2 independent experiments. Comparisons were made between samples at different time points within each species. Abbreviation: NE, nano-emulsion.

			Oil droplet size D (4, 3) (μm)			
	Bacteria	Time (days)	Coarse M9 - 25°C	NE M9 - 25°C	Coarse Broth - 25°C	NE Broth - 25°C
<i>E. coli</i> (MG1655)	yes	0	28.892 \pm 0.200 ^a	0.269 \pm 0.011 ^a	31.557 \pm 2.535 ^a	0.292 \pm 0.292 ^a
		7	26.994 \pm 0.328 ^a	0.29 \pm 0.013 ^a	33.181 \pm 2.423 ^a	0.303 \pm 0.007 ^a
	no	0	28.582 \pm 0.096 ^a	0.261 \pm 0.007 ^a	31.596 \pm 2.423 ^a	0.303 \pm 0.303 ^a
		7	25.883 \pm 0.164 ^a	0.263 \pm 0.000 ^a	33.181 \pm 0.077 ^a	0.292 \pm 0.001 ^a
<i>E. coli</i> (BW2115)	yes	0	28.316 \pm 0.423 ^a	0.248 \pm 0.007 ^a	29.654 \pm 0.080 ^a	0.306 \pm 0.012 ^a
		7	27.539 \pm 0.442 ^a	0.285 \pm 0.003 ^a	29.894 \pm 0.097 ^a	0.290 \pm 0.001 ^a
	no	0	27.062 \pm 0.322 ^a	0.255 \pm 0.016 ^a	29.908 \pm 0.028 ^a	0.307 \pm 0.015 ^a
		7	26.203 \pm 0.155 ^a	0.268 \pm 0.019 ^a	29.067 \pm 0.016 ^a	0.303 \pm 0.016 ^a
<i>E. coli</i> (JM109)	yes	0	28.842 \pm 0.094 ^a	0.285 \pm 0.007 ^a	29.468 \pm 0.994 ^a	0.317 \pm 0.003 ^a
		7	27.514 \pm 0.036 ^a	0.268 \pm 0.017 ^a	29.599 \pm 0.043 ^a	0.298 \pm 0.013 ^a
	no	0	27.809 \pm 0.157 ^a	0.244 \pm 0.004 ^a	29.411 \pm 0.093 ^a	0.294 \pm 0.005 ^a
		7	27.562 \pm 1.029 ^a	0.255 \pm 0.016 ^a	29.823 \pm 1.030 ^a	0.315 \pm 0.019 ^a
<i>E. coli</i> (MC4100)	yes	0	33.708 \pm 7.866 ^a	0.275 \pm 0.004 ^a	29.644 \pm 0.041 ^a	0.308 \pm 0.013 ^a
		7	33.807 \pm 7.153 ^a	0.285 \pm 0.008 ^a	30.025 \pm 0.204 ^a	0.299 \pm 0.010 ^a
	no	0	33.754 \pm 7.592 ^a	0.28 \pm 0.008 ^a	30.348 \pm 0.467 ^a	0.309 \pm 0.025 ^a
		7	33.57 \pm 7.307 ^a	0.277 \pm 0.005 ^a	29.583 \pm 0.008 ^a	0.294 \pm 0.003 ^a
<i>E. coli</i> (PHL644)	yes	0	27.692 \pm 1.852 ^a	0.292 \pm 0.004 ^a	35.224 \pm 0.406 ^a	0.312 \pm 0.004 ^a
		7	26.978 \pm 0.841 ^a	0.275 \pm 0.008 ^a	35.689 \pm 0.692 ^a	0.301 \pm 0.009 ^a
	no	0	28.555 \pm 0.190 ^a	0.2808 \pm 0.015 ^a	35.860 \pm 0.376 ^a	0.308 \pm 0.004 ^a
		7	25.419 \pm 0.782 ^a	0.279 \pm 0.008 ^a	33.072 \pm 5.154 ^a	0.291 \pm 0.001 ^a
<i>B. cereus</i>	yes	0	32.625 \pm 4.343 ^a	0.326 \pm 0.036 ^a	29.960 \pm 0.622 ^a	0.322 \pm 0.002 ^a
		7	32.559 \pm 3.562 ^a	0.299 \pm 0.022 ^a	29.520 \pm 0.254 ^a	0.294 \pm 0.009 ^a
	no	0	32.439 \pm 4.410 ^a	0.3085 \pm 0.005 ^a	29.685 \pm 0.021 ^a	0.308 \pm 0.013 ^a
		7	32.625 \pm 4.343 ^a	0.315 \pm 0.028 ^a	29.749 \pm 0.143 ^a	0.295 \pm 0.004 ^a
<i>S. epidermidis</i>	yes	0	31.957 \pm 0.240 ^a	0.316 \pm 0.007 ^a	29.489 \pm 0.243 ^a	0.314 \pm 0.004 ^a
		7	32.033 \pm 0.158 ^a	0.303 \pm 0.005 ^a	28.966 \pm 0.234 ^a	0.309 \pm 0.001 ^a
	no	0	32.399 \pm 0.133 ^a	0.295 \pm 0.009 ^a	29.602 \pm 0.238 ^a	0.290 \pm 0.007 ^a
		7	32.231 \pm 0.351 ^a	0.302 \pm 0.009 ^a	30.713 \pm 0.062 ^a	0.302 \pm 0.017 ^a

The data was analysed with one-way ANOVA

^a means \pm standard deviation with different letters are significantly different

3.4 Discussion

To understand the effect of droplet size on the survival of bacteria, the viability of bacteria in nano-emulsion was monitored and compared to coarse emulsion over time. The M9 minimal growth medium used as continuous phase contains minimum nutrients that can sustain possible growth but lacks the presence of amino acids, therefore bacteria can grow in the exponential phase but slowly. As opposed to being in stationary phase (non-growing), bacteria in exponential phase of growth are more susceptible to stresses (Anderl *et al.*, 2003; Matsuo *et al.*, 2011) which allows for better detection of any effects of nano-emulsion on bacterial survival. In this study, there was no significant difference observed in viability of different bacterial species in nano-emulsion compared to coarse emulsion and control M9 minimal growth medium, after 2 and 7 days. *E. coli* presented no difference in growth between nano- and coarse emulsions, and the effect was not strain dependent as no variation in responses was observed between different strains. Similarly, the reduction in counts of *B. cereus* and *S. epidermidis* was comparable between nano- and coarse emulsions. In the case of *L. acidophilus* the M9 minimal growth medium could not support its survival after day 2 and therefore this species was discontinued from this part of the study. Also, microscopic observation showed that all bacteria grew as planktonic cells and no clustering leading to colony formation was observed. Since colony formation in emulsion systems are associated with upregulation of stress genes (Prachaiyo and McLandsborough, 2003), it can be concluded that no such effects occurred in this study.

Many studies claimed that nano-emulsions enhance the activity of antimicrobials against bacteria. However, the mechanism behind this enhanced antimicrobial activity have not been clearly justified mainly due to lack in use of proper controls.

For example, TEOP and BCTP are the most commonly reported nano-emulsion formulations to possess antimicrobial activity against several species of micro-organisms including bacteria such as *E. coli*, *S. aureus* and *L. monocytogenes* (Hamouda *et al.*, 1999; Teixeira *et al.*, 2007; Buranasuksombat *et al.*, 2011). BCTP is made of soybean oil containing the antimicrobial compound cetylpyridinium chloride (CPC) and stabilised with tri-*n*-butyl phosphate and Triton X-100 (emulsifier), while TEOP is made of ethyl oleate and stabilised with Tween80 and *n*-pentanol (co-emulsifier). In 2010, Ferriera *et al.* investigated the two nano-emulsion formulations and found that for the TEOP formulation, the antimicrobial effects were due to *n*-pentanol which sits at the O/W interface as no differences in reduction of bacterial counts were observed when the bacteria were treated with the TEOP formulation compared to a solution of *n*-pentanol with the same concentration. Moreover, for the BCTP formulation it was found that the antimicrobial effect was due to CPC (water soluble, cationic surface-active agent) and its efficacy was shown to be reduced when it was incorporated into the nano-emulsion compared to as a solution with the same concentration (Ferriera *et al.* 2010). The authors argued that controls were not included in studies reporting antimicrobial activity for the BCTP and TEOP formulations and that they could have evaluated the contributions of the different components of the emulsions for the observed antimicrobial activity. Hamouda and Baker (2000) investigated the antimicrobial activity of two nano-emulsion formulations: 8N8 and W60C against *E. coli*, *Salmonella typhimurium* and *Vibrio cholera*. 8N8 is a water-in-oil nano-emulsion made of soybean oil containing CPC and stabilised with tri-*n*-butyl phosphate and Triton X-100 while W60C is a liposome made of soybean oil stabilised with Tween60, glycerol monooleate and refined soya sterols. Furthermore, both nano-emulsion formulations showed antimicrobial effects against all the bacteria; however, no testing of antimicrobial

effects of the individual components of the nano-emulsion formulation was carried out. In this case, it is not possible to attribute such effects to high surface tensions of nano-sized droplets. Thus, the process is probably not mechanical, but rather chemical. Chang *et al.* (2012) studied the antimicrobial effects of thyme oil nano-emulsion on *Zygosaccharomyces bailii*. They found that nano-emulsions made with corn and MCT oil did not exhibit any antimicrobial effects unless mixed with thyme oil. More recently, Ghost *et al.* (2014) found that sesame oil nano-emulsion possessed antimicrobial activity against *S. aureus* only when the antimicrobial compound eugenol was present in the oil phase and no such effects were occurring in the absence of eugenol. Therefore, it could be concluded that the antimicrobial activity of nano-emulsions reported in several cases in the literature can only be attributed to the antimicrobial agents that they carry, and no such activity can result from high surface tensions and cell wall diffusion of nano-sized droplets.

In many cases, antimicrobial treatments can affect bacterial cells, and although they remain alive, result in stressed and injured subpopulations, which cannot be detected with analysis by culture. In this study, the membrane integrity of the bacteria was assessed using flow cytometry combined with PI staining which is non-permeant but can penetrate cells with a compromised membrane and binds to double stranded DNA by *intercalating* between base pairs (Zhang *et al.*, 2001). According to the flow cytometry data, there was no significant increase in percentage of PI positive cells observed after incubation in nano-emulsion compared to coarse emulsion and controls in M9 medium. These results confirm that the membrane integrity of the bacteria was not affected by the nano-sized droplets and are in contrast to studies that reported extensive damage to the membrane of bacteria after exposure to nano-emulsions. Extensive disintegration of the cell membrane,

disruption to cell wall and lysis of *S. mutans* after exposure to soybean oil nano-emulsion containing CPC was observed using SEM (Karthikeyan *et al.*, 2011). Ghosh *et al.* (2013) found that exposure to basil oil nano-emulsions against *E. coli* led to deformation in bacterial membrane phospholipids (confirmed by FT-IR analysis) and stained positive with ethidium bromide (EtBr) which only stains the DNA of cells with a membrane that lost its structural integrity. Exposure to eucalyptus oil nano-emulsion led to damage of cell membrane of *S. aureus* observed using SEM (Sugumar *et al.*, 2014). The exposure of *S. aureus*, *B. subtilis*, *E. coli* and *S. cerevisiae* to nano-emulsion made with D-limonene containing the antimicrobial nisin caused extensive membrane damage observed using SEM associated with release of cellular contents evident by leakage of the cytoplasmic content measured using UV absorbance (Zhang *et al.*, 2014). In another study, exposure to oregano oil nano-emulsion led to disruption of the bacterial membrane in *L. monocytogenes*, *S. typhimurium* and *E. coli* 0157:H7 observed using SEM (Bhargava *et al.*, 2015). However, in all these studies, the antimicrobial activity of the nano-emulsion was compared to PBS, sterile water, or broth as control rather than being compared to the individual components of the nano-emulsion. In a study by Karthikeyan *et al.* (2012) reported that there were higher antimicrobial effects against biofilm and planktonic forms of *S. mutans*, *L. casei*, after a 1-minute exposure to soybean oil nano-emulsion containing CPC compared to CPC solution only more damage to the cells membrane was evident by increased fluorescence intensity of PI using fluorescence microscopy. However, no effects of the nano-emulsion without the incorporation of CPC was compared. CPC is water soluble and the lower antimicrobial activity with CPC solution would be expected since the concentration of CPC in the continuous phase of the nano-emulsion would be higher (due to the presence of the dispersed oil phase) thus bacterial cells will be exposed to a higher

concentration of CPC in nano-emulsion compared to CPC solution. Although the study showed that nano-emulsion can damage the membrane of bacteria (Karthikeyan *et al.*, 2012), it lacks use of full controls and conclusions should be interpreted with caution.

In order to investigate if nano-emulsions affect bacteria during growth and proliferation, viability was compared between O/W nano-emulsion and coarse emulsion made with tryptic soy broth as continuous phase. Once again, there was no significant difference in growth of bacteria between nano- and coarse emulsion. Growth patterns were similar regardless of species and strains and comparable to tryptic soy broth (control). Also, bacteria grew as planktonic cells and no clustering leading to colony formation was observed as response to stress. Furthermore, the *E. coli* strain PHL644 which is a potent biofilm former that overexpress the protein adhesin curli (surface attachment structures) (Vidal *et al.*, 1998; Perni *et al.*, 2013) maintained its planktonic form within the nano-emulsion. These results support a study conducted by Naïtali *et al.* (2009) where the growth kinetics of *L. monocytogenes* was not affected by incubation in nano-emulsions. In contrast, it was observed that as opposed to growing in planktonic form, *L. monocytogenes* were constrained to grow as colonies in O/W emulsions with higher oil phase concentrations (>80% vs 30 or 70%) and smaller droplet size (2µm vs 15 or 25µm) (Brocklehurst *et al.*, 1995). The authors argued that in such emulsions the oil droplets were sufficiently close-packed and viscous to prevent the mobility of the bacteria forcing growth in colonies, therefore it was a response to space and not a biological response of cells to interaction with nano-sized droplets. Also, the growth rates of bacteria were reduced due to restricted diffusion of nutrients and oxygen or accumulation of waste, but this only occurred at lower pH (5 vs 7). However, in this

study the conditions were not similar as the oil phase concentration was 40% and the pH value of the emulsions during inoculation were around ~7.3.

In order to investigate the effect of oil droplet size in O/W emulsions in combination with antimicrobial components, caprylic acid (CA) was added in the oil phase. Since CA is minimally soluble in water and soluble in fat, its antimicrobial activity should be affected by the surface area of the oil phase. The antimicrobial activity of O/W emulsions containing CA was investigated in varying concentration of Tween80. The antimicrobial activity of 0.5% CA was enhanced in emulsions (CA), and resulted to comparable bacterial reductions with 1% CA in non-homogenised sample after 8 and 24 hours. These results are in agreement with reports on higher activity of antimicrobial oils in emulsions compared to bulk form, including eucalyptus oil nano-emulsion against *B. cereus*, *S. aureus* and *E. coli* (Sugumar *et al.*, 2013) thyme oil nano-emulsion against *E. coli* O157:H7, *L. monocytogenes* and *S. enteritidis* (Wu *et al.*, 2014; Xue *et al.*, 2015), *Thymus daenensis* essential oil against *E. coli* (Moghimi *et al.*, 2016a), sage oil (*Salvia officinalis*) (Moghimi *et al.*, 2016b) anise oil against *L. monocytogenes* and *E. coli* O157:H7 (Topuz *et al.*, 2016), peppermint oil (PO) against *S. aureus* and *L. monocytogenes* (Liang *et al.*, 2012). The EO's possess antimicrobial properties and the increasing surface area of the oil interface in these studies, enhances the activity on bacterial membrane compared to bulk form, without however any EO-emulsion synergistic effect being observed, and therefore nano-size globules in emulsion do not directly contribute to the activity. The CA molecule is oriented so that the carboxyl group protrudes into the aqueous phase, while the hydrocarbon tail is in the oil phase (Andersson *et al.*, 2014). Therefore, it would be expected that the higher surface area in emulsion increases the amount of CA in contact with the bacterial membrane compared to non-homogenised form. *S.*

epidermidis was more susceptible than *E. coli* in non-homogenised sample containing 0.5% CA, and its viability significantly ($P < 0.05$) decreased (~ 4 -log CFU/ml and < 2 -log CFU/ml at 8 and 24h respectively). The increased susceptibility of *S. epidermidis* compared to *E. coli* in non-homogenised sample containing 0.5% CA could be due to the lack of the outer membrane in Gram-positive bacteria which provides extra protection to the peptidoglycan cell wall in Gram-negative bacteria. These results corroborate studies reporting that Gram-positive bacteria are more sensitive to the antimicrobial effects of CA (Nair *et al.*, 2005) and other fatty acids (Monk *et al.*, 1996) than Gram-negative bacteria.

The effect of Tween80 concentration on the antimicrobial activity of O/W emulsions containing CA was investigated. Interestingly, the CA antimicrobial activity was evident for emulsions composed with 1% Tween80 but was not in samples with 8% Tween80. To ensure that these results were not due to differences in pH, the pH was measured after 24 hours and all the samples had \sim pH 6-6.5 (results not shown). However, no differences between nano- and coarse emulsion were observed, and the responses were comparable for *E. coli* (MG1655) and *S. epidermidis*. These results indicate that the antimicrobial effects are driven by formulation (i.e. Tween80 concentration) and not size of oil droplets. The concentration of Tween80 can affect the efficacy of antimicrobials within nano-emulsions (Donsi *et al.*, 2011; Terjung *et al.*, 2012). When the concentration of hydrophilic surfactants increases in the continuous phase they form condensed interfacial films (Tadros, 2013). Since Tween80 is a non-ionic surfactant that stabilises the emulsion by steric repulsion, its increase in concentration could prevent contact of CA with the bacterial membrane at the O/W interface.

Overall, the antimicrobial activity of CA was similar in nano- and coarse emulsions. These results are in agreement with previous work on lemon myrtle in soybean oil nano-emulsion against *E. coli*, *L. monocytogenes*, *Salmonella typhimurium*, *P. aeruginosa* and *B. cereus* (Buranasuksombat *et al.*, 2011) and cinnamaldehyde against *E. coli* (Bilbao-Sainz *et al.*, 2013), suggesting no synergistic effect. Therefore, the antimicrobial effect of nano-emulsions could be considered a derivative of antimicrobials and their delivery through nano-sized droplets. However, a recent study reported the antimicrobial effect of anise oil (AO) nano-emulsion on *E. coli* and *L. monocytogenes* to be higher than AO coarse emulsion (Topuz *et al.*, 2016) due to higher surface area. Donsi *et al.* (2011) found that O/W emulsions with smaller droplets have less antimicrobial effects compared to O/W emulsions with larger droplets due to mechanical stresses caused by the high-pressure homogenisation (HPH) process when forming nano-emulsions resulting in degradation of the antimicrobial agents such as phytophenols. In this study, it may be possible that the surface area provided by the coarse emulsion was enough to allow all the CA molecules to orient at the O/W interface comparably to nano-emulsion. Furthermore, the formation of nano-emulsion by ultrasonication generates heat which may affect the antimicrobial activity of CA. Pestana *et al.* (2015) showed that the amount of CA in milk samples was diminished after pasteurisation and ultra-high temperature (UHT) sterilisation.

All O/W emulsions remained stable during the incubation period. The oil droplet size $[D(4, 3)]$ and changes in creaming stability (data not shown) with or without bacteria in the continuous phase was monitored over time (24 hours) with no significant differences observed. Furthermore, there was no flocculation and aggregation of the oil droplets observed with any of the bacterial strains regardless of their surface

characteristics. The most hydrophobic strains including *E. coli* (JM109), *S. epidermidis* and *B. cereus* resided within the continuous phase and did not aggregate around the oil droplets. Similarly, the stability of O/W emulsions with CA was not affected by the presence of bacteria. Ly *et al.* (2006) found that the stability of O/W emulsions with bacteria was strain dependent and the negatively charged *Lactococcus lactis* (LLD16) provoked creaming, flocculation, and aggregation by surrounding the positively charged oil globules whereas the positively charged *L. lactis* (LLD18) caused no such effects. In another study, it was shown that as opposed to the less negatively charged *E. coli* strain E21, the more negatively charged *E. coli* JM109 promoted faster creaming rates, coalescence and flocculation of O/W emulsions containing positively charged oil globules (Li *et al.*, 2001). In this study, the oil globules in the nano-emulsion were stabilised by a non-ionic surfactant (Tween80), hence, the absolute magnitude of the droplet charge is very low (McClements, 2011; Tang *et al.*, 2012). Since the bacterial membranes were found to be negatively charged and the oil droplets in all the O/W emulsions were less negatively charged, thus they repel each other, and bacterial cells will remain in the aqueous continuous phase. Therefore, the findings in this work on the antimicrobial activity over time, could not have been affected by changes in O/W emulsion stability.

3.5 Conclusion

The literature review identified contradictory results regarding the consistency and mechanism of antimicrobial activity reported for nano-emulsions. In this study reducing the size of oil droplets in O/W emulsions to the nano-scale had no direct effect on the viability and growth of bacteria when no antimicrobial agents were added, and flow cytometry showed that the membrane integrity was intact.

Contradiction seems to come from studies suggesting that nano-emulsions possess antimicrobial properties due to high surface tensions and cell wall diffusion of the nano-sized droplets, however, many of these studies were found to lack appropriate controls to test the action of individual components of the nano-emulsion or the action of the nano-emulsion without active ingredients. Therefore, some of the findings that attribute direct antimicrobial activity to nano-emulsions should be taken with caution, and further work is needed before concluding. In contrast, there is strong evidence that O/W nano-emulsions present higher antimicrobial activity due to increased area of O/W interface; however, the case study based on CA did not show increased antimicrobial activity in nano- compared to coarse emulsion. Therefore, it is indicated that these responses should not always be expected, and the antimicrobial effect of nano-emulsions depends on the antimicrobial agent and is affected by the formulation. Nano-emulsions remain an extremely promising asset in food formulation applications and they are known to promote stability, improve sensory perception, and enhance food functionality. In contrast, their manufacturing requires more energy. Therefore, their antimicrobial capability must be fully realised for assessing the benefits of application.

CHAPTER 4: IMPACT OF BACTERIA ON THE STABILITY OF DOUBLE $W_1/O/W_2$ EMULSIONS

4.1 Introduction

In many applications, the formulation of a highly stable $W_1/O/W_2$ emulsion is desirable. Many studies have investigated the stability of $W_1/O/W_2$ emulsions over storage. The stability of $W_1/O/W_2$ emulsions depends on the amount and type of the surfactants added (Ficheux *et al.*, 1998), osmotic pressure balance (between inner W_1 and outer W_2 phases) (Bou *et al.*, 2014), storage temperature (Cofraides *et al.*, 2013) viscosity of the oil phase (Bonnet *et al.*, 2009) and surface interaction of other particles with oil globules interface (e.g. surface charge) (Viriyakitpattana and Sunintaboon, 2016). Cofrades *et al.* (2013) assessed the stability of $W_1/O/W_2$ emulsions by globule size measurements and creaming stability tests after thermal treatment and found that the $W_1/O/W_2$ emulsions had good thermal stability and showed no significant changes in mean globule size and creaming height during the one-week storage at chilling temperature.

The applicability of $W_1/O/W_2$ emulsions can be determined by their encapsulation efficiency which depends on the formulation. Encapsulation efficiency is expressed as an extent of entrapment of hydrophilic compounds in W_1 . There is a correlation between stability and encapsulation efficiency of $W_1/O/W_2$ emulsions. It has been reported that the stability and encapsulation efficiency is affected by the type and concentration of surfactants used to stabilise the $W_1/O/W_2$ emulsion (Koberstein-Hajda and Dickinson, 1996; Hattrem *et al.*, 2014). Moreover, both stability and encapsulation efficiency can be enhanced in $W_1/O/W_2$ emulsions by increasing the viscosity of the W_1 phase (Yan *et al.*, 1992), incorporating molecules in the phases

including proteins such as bovine serum albumin (BSA) (Garti *et al.*, 1994), whey protein (Iqbal *et al.*, 2013), sodium caseinate (Fechner *et al.*, 2007; Giroux *et al.*, 2013) and faba bean proteins (Koberstein-Hajda and Dickinson, 1996), electrolytes (Rosano *et al.*, 1998) and natural polymers such as gum arabic (Zhang and Reineccius, 2016) and starch (Matos *et al.*, 2013, Mun *et al.*, 2014).

Depending on the species and strain, bacteria with opposite surface charge to the emulsion droplet will destabilise the structure of O/W emulsion and tend to aggregate around the droplets (Ly *et al.*, 2006). *Escherichia coli* can metabolize glucose and grow within water droplets of W/O emulsion and excrete products that diffuse through the oil phase and into surrounding water droplets creating an osmotic mismatch leading to water flux and droplet shrinkage at rates depending on the strain, species and number of the microbial cells (Boitard *et al.*, 2012). As opposed to growing in planktonic form, Gram negative and Gram positive bacteria were constrained to grow as colonies in O/W emulsions when the oil phase concentration was increased (Parker *et al.*, 1995) and the oil droplet size was decreased (Brocklehurst *et al.*, 1995).

However, there is a lack in studies investigating the stability of $W_1/O/W_2$ emulsions in the presence of bacteria. In this study, the stability of $W_1/O/W_2$ emulsions in the presence of *E. coli* (in W_1 phase) and/or *L. acidophilus* (in W_2 phase) was monitored and the encapsulation efficiency of the bacteria was determined during 7-day storage. Also, the viability of encapsulated *E. coli*-GFP was assessed during 7-day storage. The spatial distribution and localisation of *E. coli*-GFP and *L. acidophilus* within the $W_1/O/W_2$ emulsions was observed using fluorescence microscopy.

4.2 Materials and Methods

4.2.1 Materials

The water-soluble surfactant Tween80 was purchased from Sigma-Aldrich (United Kingdom). The oil soluble surfactant PGPR was provided by Danisco (Denmark). Sunflower oil (food grade) was purchased from a local retailer (United Kingdom). The stain DAPI was purchased from Sigma-Aldrich (United Kingdom). Nucleopore black polycarbonate membrane (13mm diameter; 2.0 μm pore size) and stainless steel 13mm Swinney filter holder was purchased from Millipore (United Kingdom). Tryptic soy agar (Oxoid Ltd. CM0131), tryptic soy broth (Oxoid Ltd. CM0129), nutrient agar (Oxoid Ltd. CM0003), *de Man, Rogosa and Sharpe* (M.R.S) agar (OXOID CM0359) and broth (OXOID CM0361) were purchased from Fisher Scientific (United Kingdom).

4.2.2 Microbial cultures

Escherichia coli strain K-12, MG1655 (ATCC 47076) and its derivative *E. coli* SCC1 (MG1655 P_{A1/04/03}-*gfp*mut3*, expressing green fluorescent protein (GFP) from the chromosome and subsequently referred to as *E. coli*-GFP) (Miao *et al.*, 2009) were maintained on tryptic soy agar petri dishes at 4°C. *Lactobacillus acidophilus* (ATCC 4356) was maintained on M.R.S agar petri dishes at 4°C. *E. coli* cells were transferred into 50 ml of tryptic soy broth in Erlenmeyer flask (250 ml), incubated at 37°C for 24 hours shaking at 150 rpm and sub-cultured to 50 ml of tryptic soy broth in Erlenmeyer flask (250 ml) for a further 2 hours. *L. acidophilus* cells were transferred into 50 ml of M.R.S broth in Erlenmeyer flask (50 ml), incubated at 37°C for 42 hours and sub-cultured to 50 ml of M.R.S broth in Erlenmeyer flask (50 ml) for a further 12 hours. For obtaining cells in the exponential phase cells were harvested

by centrifugation (10,000 g, 10 minutes) using 15 ml Falcon tubes and washed in PBS (phosphate buffered saline) buffer solution twice.

4.2.3 Preparation of $W_1/O/W_2$ emulsions

Double $W_1/O/W_2$ emulsions were prepared using a high shear mixer homogeniser (Silverson L5M) at room temperature using a two-step emulsification process (Appendix, Fig A6). In the first step, primary W_1/O emulsions were made. An oil phase was prepared by dissolving 1% or 2 wt% PGPR in sunflower oil. The inner aqueous phase (W_1) consisting of de-ionised water was emulsified (120 seconds) into the oil phase ($W_1:O$ phase ratio of 20:80 or 40:60) mixing at 3000 rpm. For microbial-encapsulation de-ionised water containing the washed bacteria (10^9 or 10^8 CFU/ml) was used as W_1 . In the second step $W_1/O/W_2$ emulsion was made. The outer aqueous phase (W_2) was prepared by dissolving Tween80 (5 wt%) in de-ionised water at 60°C for approximately 15 minutes. The previously prepared primary W_1/O emulsion was emulsified (60 seconds) into W_2 to form the $W_1/O/W_2$ emulsion ($W_1:O:W_2$ ratio of 20:80) mixing at 1800 rpm. For microbial-inoculation *L. acidophilus* (10^6 CFU/ml) was added to the W_2 phase. Finally, the whole 100 ml of $W_1/O/W_2$ emulsion was transferred into sterile Erlenmeyer flask (500 ml) and incubated at 25°C for a 7-day period shaking at 100 rpm to ensure homogenised mixing.

4.2.4 Fluorescent and optical imaging of $W_1/O/W_2$ emulsions with and without bacteria

The double $W_1/O/W_2$ emulsions with or without *E. coli*-GFP and *L. acidophilus* were observed using optical and fluorescent microscopy (Zeiss Axioplan) at room temperature. For optical microscopy imaging the sample was placed on a microscope slide and the image was acquired under objective lens 10x magnification

with a digital colour camera system Motic Motacam 10 using a 10 megapixel CMOC camera via Motic Images Plus video acquisition software. For fluorescent microscopy imaging the samples were transferred into 1.5 ml Eppendorf tubes and stained by adding DAPI (4 μ l/ml) and incubated in the dark for 30 minutes. The stained sample was placed on a microscope slide and gently covered with a cover slip. The image was acquired under objective lens 100x magnification (oil immersion) with a digital camera system AxioCam ICm1 using a 1.4 megapixel monochrome CCD camera via AxioVision Software (Zeiss). The samples were observed at room temperature using a fluorescent microscope (Zeiss AxioLab) equipped with a mercury arc lamp and the emission was observed at 461 nm (DAPI) and 509 nm (GFP). Micrographs were overlaid using analysis software (ImageJ).

4.2.5 Determination of bacterial viability

Bacterial cell counts were made immediately after preparation and as a function of storage time using serial dilutions in *PBS* and plating on tryptic soy agar using the Miles & Misra technique (Miles *et al.*, 1938). Colony forming units per millilitre (CFU/ml) were then calculated.

4.2.6 Characterisation of $W_1/O/W_2$ emulsion stability

The stability of the $W_1/O/W_2$ emulsions was monitored by measuring the oil globule size [D (4, 3)] and creaming thickness during incubation.

4.2.6.1 Measurement of oil globule size of $W_1/O/W_2$ emulsions

The particle size distribution of oil globules in the $W_1/O/W_2$ emulsion was measured immediately after preparation and as a function of storage time using a laser diffraction particle size analyser (Malvern Mastersizer 2000, Malvern Instrument Ltd,

Worcestershire, UK), equipped with a He-Ne laser ($\lambda = 633 \text{ nm}$). This procedure was done as in Section 3.2.6.1.

4.2.6.2 Observation of phase separation

The cream height fraction of the $W_1/O/W_2$ emulsion was measured immediately after preparation and as a function of storage time. This procedure was done as in Section 3.2.6.2.

4.2.7 Measuring encapsulation efficiency (%) of *E. coli* (MG1655) in $W_1/O/W_2$ emulsions

Due to the differences in density between the W_2 phase and the oil globules creaming or phase separation occurs when $W_1/O/W_2$ emulsion is un-mixed and it partitions into a serum phase (W_2 phase) and a cream layer (containing oil globules) (Robins *et al.*, 2002). The number of *E. coli* cells in the serum phase was measured immediately after preparation and as a function of storage time. Five millilitre sample of $W_1/O/W_2$ emulsion was collected and the serum phase was removed using a 5ml graduated syringe. *E. coli* MG1655 in serum phase or de-ionised water (control) was stained by adding DAPI (4 μ l/ml) and PI (4 μ l/ml) and incubated in the dark for 30 minutes. A nucleopore black polycarbonate membrane filter with 0.2 μ m pore size and 13mm diameter was mounted shiny side, uppermost on a membrane Swinnex filter holder (Millipore) and the stained solution was passed through the membrane filter by injecting slowly with a 5ml graduated syringe. The membrane filter was placed shiny side uppermost on a glass slide and a drop of immersion oil added to its surface and gently covered with a cover slip. The cells were then counted under objective lens of 100x magnification with a fluorescent microscope (Zeiss Axiolab) equipped with a mercury arc lamp and the emission was observed at 461nm (DAPI)

and 645nm (PI). For each filter, ten microscopic (0.1mm x 0.1mm) fields were randomly selected and all cells within each field were counted and the average number of cells per field was calculated (Hobbie *et al.*, 1977). The serum phase volume was adjusted to yield a minimum of 400 total cell counts in 10 counting fields. The total number of *E. coli* cells from each sample of serum phase was calculated by multiplying the total number of fields with the average number of cells per field. The encapsulation efficiency of *E. coli* cells was calculated by the following equations:

$$\text{Encapsulation efficiency} = ((N_0 - N) / N_0) \times 100\% \quad (4.1)$$

$$\text{Release} = (\text{Log}_{10} N_T - \text{Log}_{10} N) \quad (4.2)$$

Where N is the number of unencapsulated bacterial cells immediately after forming the $W_1/O/W_2$ emulsion, N_0 displays the bacterial cells before encapsulation and N_T displays the unencapsulated bacterial cells after incubation period.

4.2.8 Statistical analysis

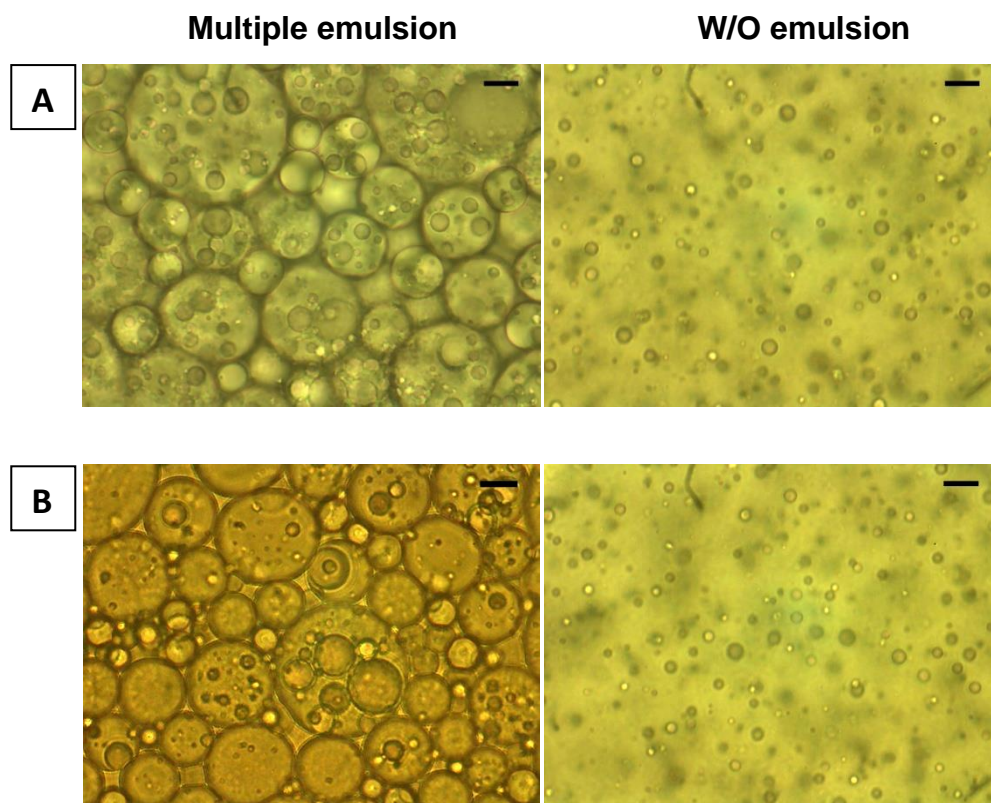
Statistical analysis was done as described in Section 3.2.10 and each experiment was conducted in triplicate (N=3).

4.3 Results

4.3.1 Effect of *E. coli*-GFP on W_1/O emulsion

The effect of concentration of bacteria on the stability of the primary W_1/O emulsion was investigated. When the primary W_1/O emulsion contained 40% W_1 and 2% PGPR, high concentration of *E. coli*-GFP (10^9 CFU/ml) prevented emulsion at 40% W_1 and 2% PGPR W_1/O to form; however, primary W_1/O emulsions could form at low concentration of *E. coli*-GFP (10^8 CFU/ml) (Figure 3.1A). The formation of primary W_1/O emulsion did not occur at a high concentration of *E. coli*-GFP (10^9

CFU/ml) with 40% W_1 but could form at 20% W_1 (Figure 3.1B). When the PGPR concentration was reduced to 1%, the W_1/O emulsion did not form at low concentration of *E. coli*-GFP (10^8 CFU/ml) but could form without the presence of *E. coli*-GFP (Figure 3.1C). To find out whether this phenomenon was due to the metabolic activity of bacteria, dead *E. coli*-GFP cells (10^9 CFU/ml) were added to 40% W_1 and homogenised with the oil phase containing 2% PGPR. In the presence of dead *E. coli*-GFP cells the formation of primary W_1/O emulsion did not occur at 10^9 CFU/ml but could form at 10^8 CFU/ml (Figure 3.1D) showing that metabolic activity played no role in destabilising the primary W_1/O emulsion. These results suggest that the presence of *E. coli* cells (live or dead) within the W_1 phase affected the formation of the primary W_1/O emulsion during the first step of the homogenisation process and this depends on the concentration of *E. coli*-GFP and/or PGPR.



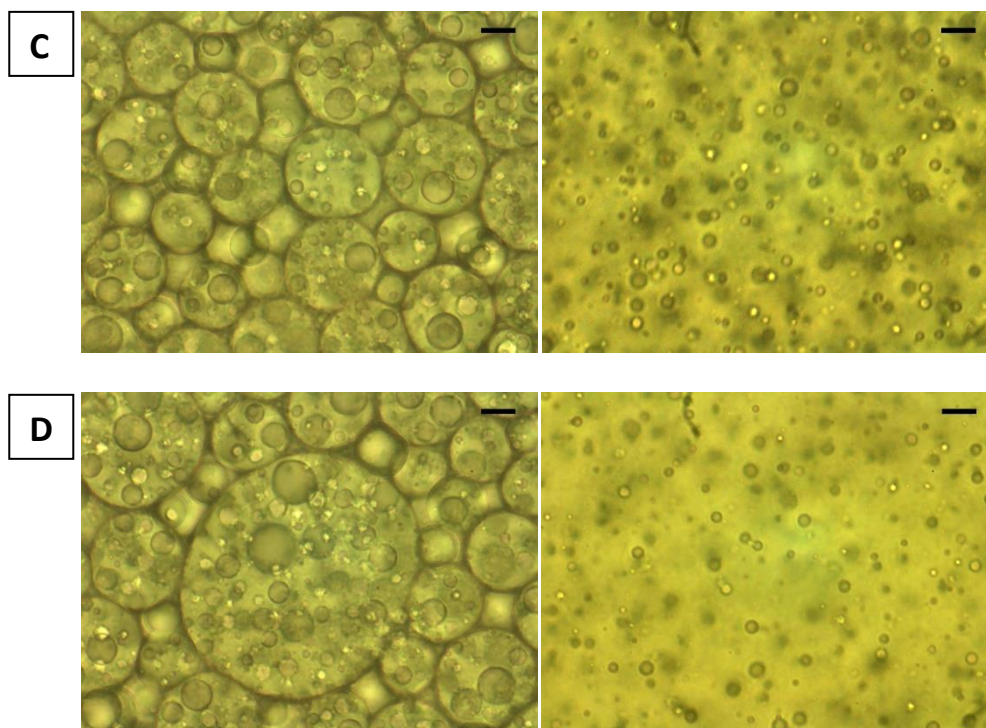


Figure 4.1 Single W_1/O emulsions prepared with different concentrations of PGPR or concentrations of *E. coli*-GFP. The images on the left show the multiple emulsions and the images on the right show the W_1/O emulsions. The W_1/O emulsions were prepared with inner-phase (W_1) volume percentage of 40% or 20% containing *E. coli*-GFP and stabilised with PGPR. The formulations were as follows: A) 2% PGPR containing live cells at 10^9 CFU/ml in 40% W_1 (left) and 10^8 CFU/ml in 40% W_1 (right); B) 2% PGPR containing live cells at 10^9 CFU/ml in 40% W_1 (left) and in 20% W_1 (right); C) 1% PGPR containing live cells at 10^8 CFU/ml in 40% W_1 (left) and no *E. coli*-GFP in 40% W_1 (right); D) 2% PGPR containing dead cells at 10^9 CFU/ml in 40% W_1 (left) and 10^8 CFU/ml in 40% W_1 (right); Scale bar: 10 μ m.

4.3.2 Primary W_1/O emulsion characterization

The size of the W_1 droplets can influence the stability of $W_1/O/W_2$ emulsions (Weiss and Muschiolik, 2007). In this study, the homogenisation conditions were such that the average mean size distribution [D (4, 3)] of W_1 droplets was comparable (3-5 μ m; Appendix, Figures A2 and A3) in all primary W_1/O formulations. *E. coli*-GFP was successfully encapsulated within the W_1 droplets of the primary W_1/O emulsion (Appendix, Figure A4).

4.3.3 Encapsulation efficiency (%) of *E. coli*-GFP cells in $W_1/O/W_2$ emulsions

During encapsulating bacteria in $W_1/O/W_2$ globules some cells may not be successfully entrapped within the globules remaining in the continuous W_2 phase which in turn determines the applicability of such systems. Encapsulation efficiency, expressed as an extent of entrapment of *E. coli*-GFP cells in different $W_1/O/W_2$ emulsion formulations, was calculated by measuring the bacterial cell counts before and after the encapsulation process. The results indicated successful entrapment of viable bacterial cells (>99.9%) in all the prepared $W_1/O/W_2$ emulsions (Figure 4.2). The encapsulation efficiency was significantly ($P < 0.05$) reduced after 3 and 7 days (93.4% and 92.6% respectively). Furthermore, there was no significant difference in encapsulation efficiency at day 3 compared to day 7.

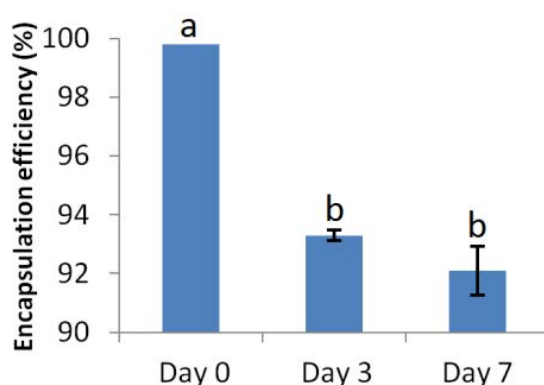


Figure 4.2 Encapsulation efficiency (%) of *E. coli*-GFP in $W_1/O/W_2$ emulsion at 0, 3 and 7 days. The $W_1/O/W_2$ emulsion was made with 20% W_1 and stabilised with 5% Tween80 in W_2 and 2% PGPR in the oil phase. Results are taken from a minimum of 3 independent experiments. Bars represent mean \pm SEM taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different ($P < 0.05$). The data was analysed with one-way ANOVA.

4.3.4 Changes in oil globule size during osmotic balance alteration and storage

To understand how the stability of $W_1/O/W_2$ globules is affected by the presence of the two species of bacteria the change in oil globule size ($D(4, 3)$) was measured over time. The effect of having *E. coli*-GFP in W_1 , *L. acidophilus* in W_2 or both *E. coli*-GFP in W_1 and *L. acidophilus* in W_2 simultaneously present in the $W_1/O/W_2$ emulsion on the size ($D(4, 3)$) of the oil globules (Figure 4.3) was measured.

The presence of bacteria in W_1 had no effect on the $D(4, 3)$ during the incubation period. There was no significant difference in $D(4, 3)$ when both *E. coli*-GFP in W_1 and *L. acidophilus* in W_2 were simultaneously present in the $W_1/O/W_2$ emulsion compared to no bacteria present (control). There was no significant difference in $D(4, 3)$ when *E. coli*-GFP in W_1 or *L. acidophilus* in W_2 are present in the $W_1/O/W_2$ emulsion compared to no bacteria present (control) (Figure 4.3). These results indicate that the $D(4, 3)$ is not affected by the presence of the two species of bacteria during storage.

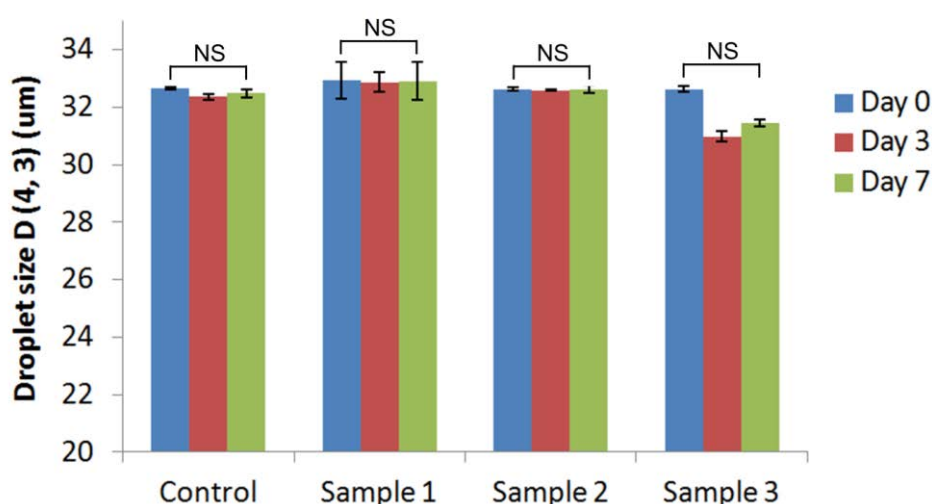


Figure 4.3 Mean oil globule diameter size (μm) of $W_1/O/W_2$ emulsions by light scattering [$D(4, 3)$] at 0, 3 and 7-day incubation at 25°C . The $W_1/O/W_2$ emulsions were prepared with 20% W_1 and stabilised with 5% Tween80 in W_2

and 2% PGPR in the oil phase: Control (no bacteria), Sample 1: *E. coli*-GFP in the inner water phase, Sample 2: *L. acidophilus* in the outer phase, Sample 3: *L. acidophilus* in the outer phase and *E. coli*-GFP in the inner water phase. Results are taken from a minimum of 3 independent experiments. The data was analysed with one-way ANOVA.

4.3.5 Changes in creaming behaviour during osmotic balance alteration and storage

Changes in creaming height were measured to determine if the creaming behaviour of the $W_1/O/W_2$ emulsion is affected by the presence of the two species of bacteria. The effect of having *E. coli*-GFP in W_1 , *L. acidophilus* in W_2 or both *E. coli*-GFP in W_1 and *L. acidophilus* in W_2 simultaneously present in the $W_1/O/W_2$ emulsion on creaming height of the $W_1/O/W_2$ emulsions (Figure 4.4) was measured.

The presence of bacteria in W_1 had no effect on the creaming height during the incubation period. There was no significant difference in creaming height when both *E. coli*-GFP in W_1 and *L. acidophilus* in W_2 were simultaneously present in the $W_1/O/W_2$ emulsion compared to no bacteria present (control) (Figure 4.4). There was no significant difference in creaming height when *E. coli*-GFP in W_1 or *L. acidophilus* in W_2 is present in the $W_1/O/W_2$ emulsion compared to no bacteria present (control) (Figure 4.4). These results indicate that creaming behaviour is not affected by the presence of the two species of bacteria during storage.

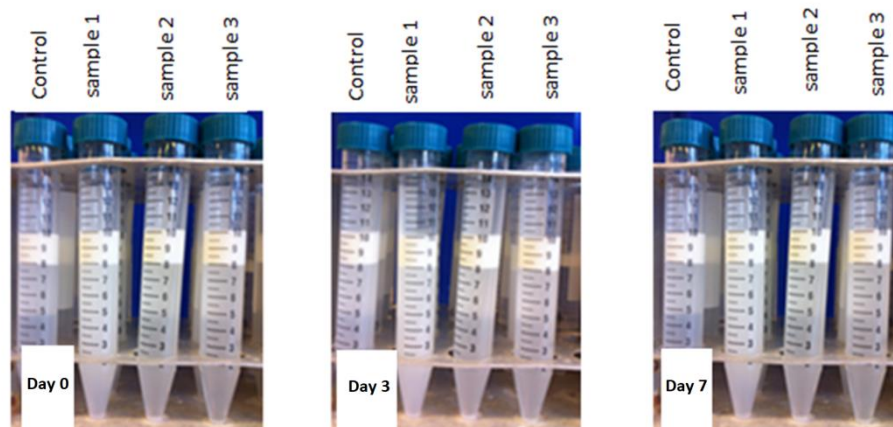


Figure 4.4 Creaming stability measurements in falcon tubes of $W_1/O/W_2$ double emulsions at 0, 3 and 7-day incubation at 25°C. The $W_1/O/W_2$ emulsions were prepared with 20% W_1 and stabilised with 5% Tween80 in W_2 and 2% PGPR in the oil phase: Control (no bacteria), Sample 1: *E. coli*-GFP in the inner water phase, Sample 2: *L. acidophilus* in the outer phase, Sample 3: *L. acidophilus* in the outer phase and *E. coli*-GFP in the inner water phase. Results are taken from a minimum of 3 independent experiments.

4.3.6 Microscopic observation of *E. coli*-GFP and/or *L. acidophilus*

The $W_1/O/W_2$ emulsions were observed using light and fluorescence microscopy to study the emulsion structure and localisation of the two species of bacteria within the emulsions (Figure 4.5-4.8). In the presence or absence of *E. coli*-GFP and/or *L. acidophilus* $W_1/O/W_2$ emulsions showed no flocculation and the inner W_1 phase was still present during storage (Figure 4.5-4.8). Although mainly present in the inner W_1 phase, some *E. coli*-GFP cells were observed in the W_2 phase at day 3 and 7 (Figure 4.6B-C and 4.8B-C). *L. acidophilus* stayed in the W_2 phase with no interaction with the oil globules observed (Figure 4.7 and 4.8). However, *L. acidophilus* cells had an elongated morphology at day 3 and 7 (4.7B-C and 4.8B-C).

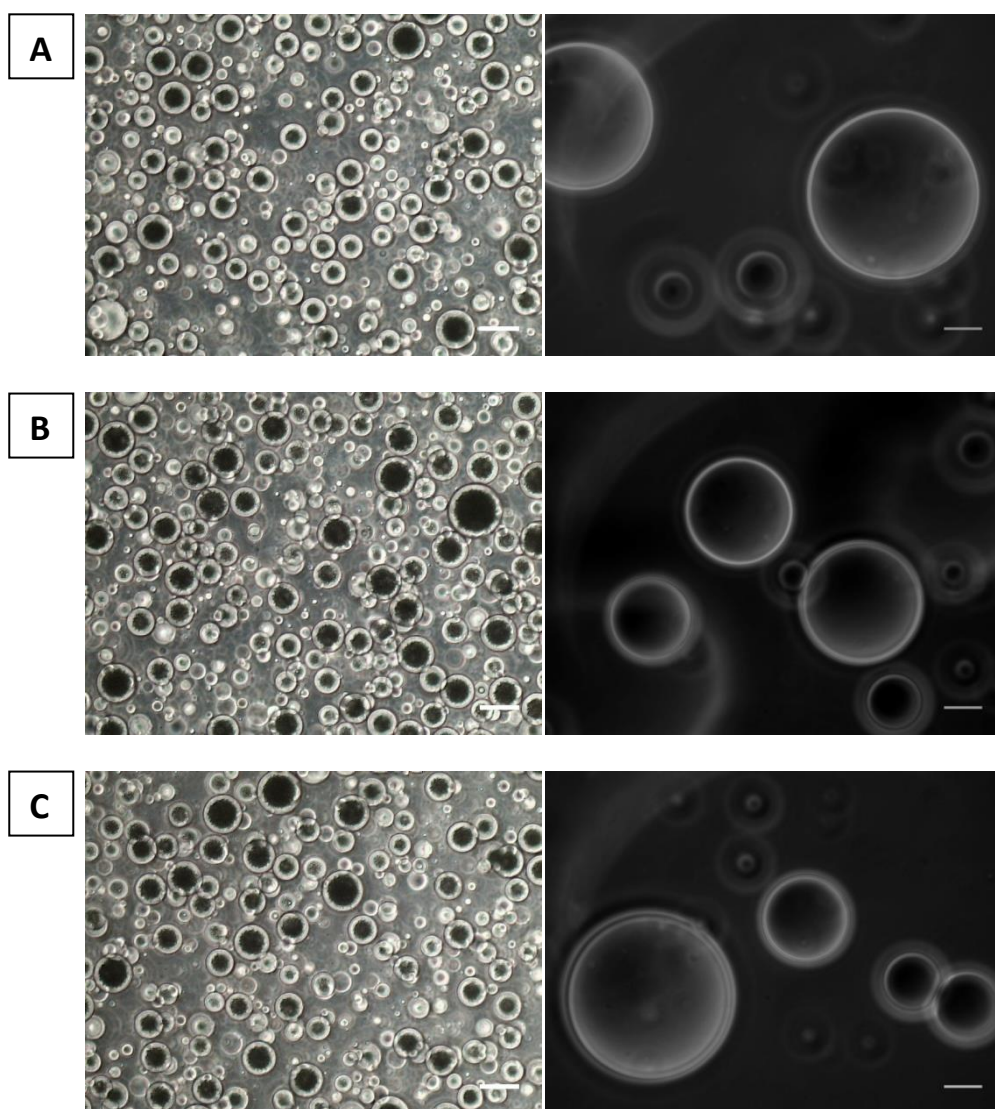


Figure 4.5 Micrographs of $W_1/O/W_2$ double emulsions using optical (left) and fluorescence (right) microscopy at 0, 3 and 7 days (A, B, and C respectively). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 20% and stabilised with 5% Tween80 in W_2 and 2% PGPR in the oil phase. Scale bar: 50 μ m (left) and 10 μ m (right).

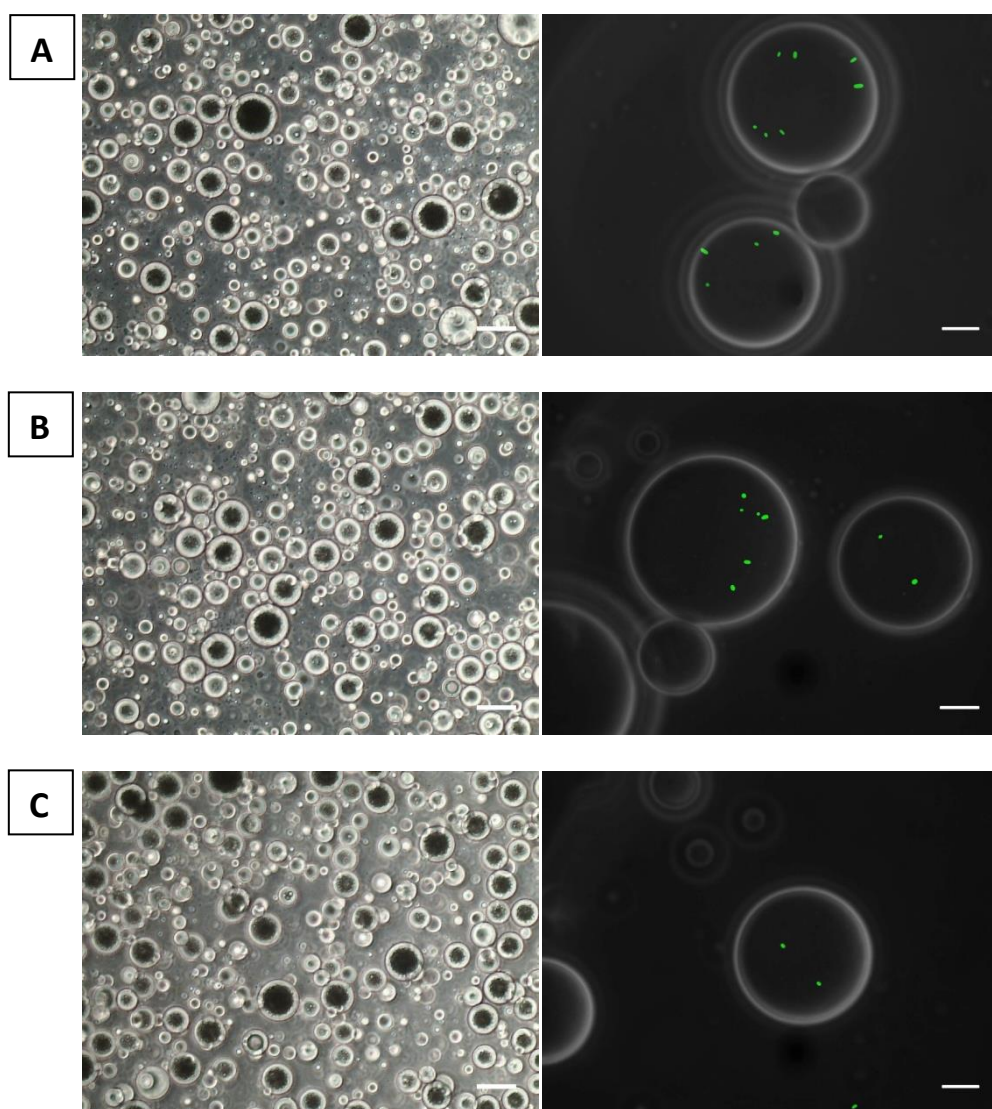


Figure 4.6 Micrographs of $W_1/O/W_2$ double emulsions using optical (left) and fluorescence (right) microscopy at 0, 3 and 7 days (A, B, and C respectively). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 20% containing *E. coli*-GFP in the inner W_1 phase and stabilised with 5% Tween80 in W_2 and 2% PGPR in the oil phase. Scale bar: 50µm (left) and 10µm (right).

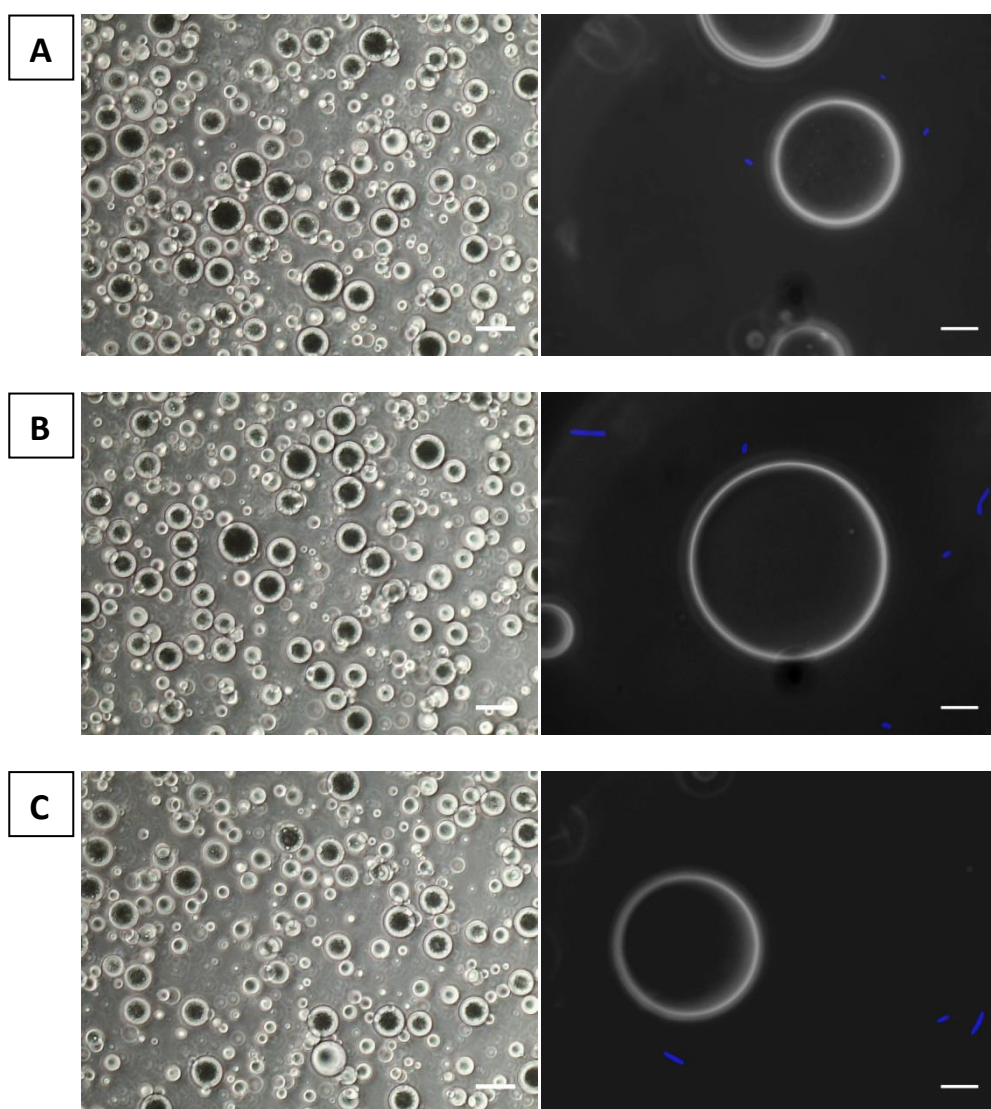


Figure 4.7 Micrographs of $W_1/O/W_2$ double emulsions using optical (left) and fluorescence (right) microscopy at 0, 3 and 7 days (A, B, and C respectively). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 20% containing *L. acidophilus* in the outer W_2 phase and stabilised with 5% Tween80 in W_2 and 2% PGPR in the oil phase. Scale bar: 50 μ m (left) and 10 μ m (right).

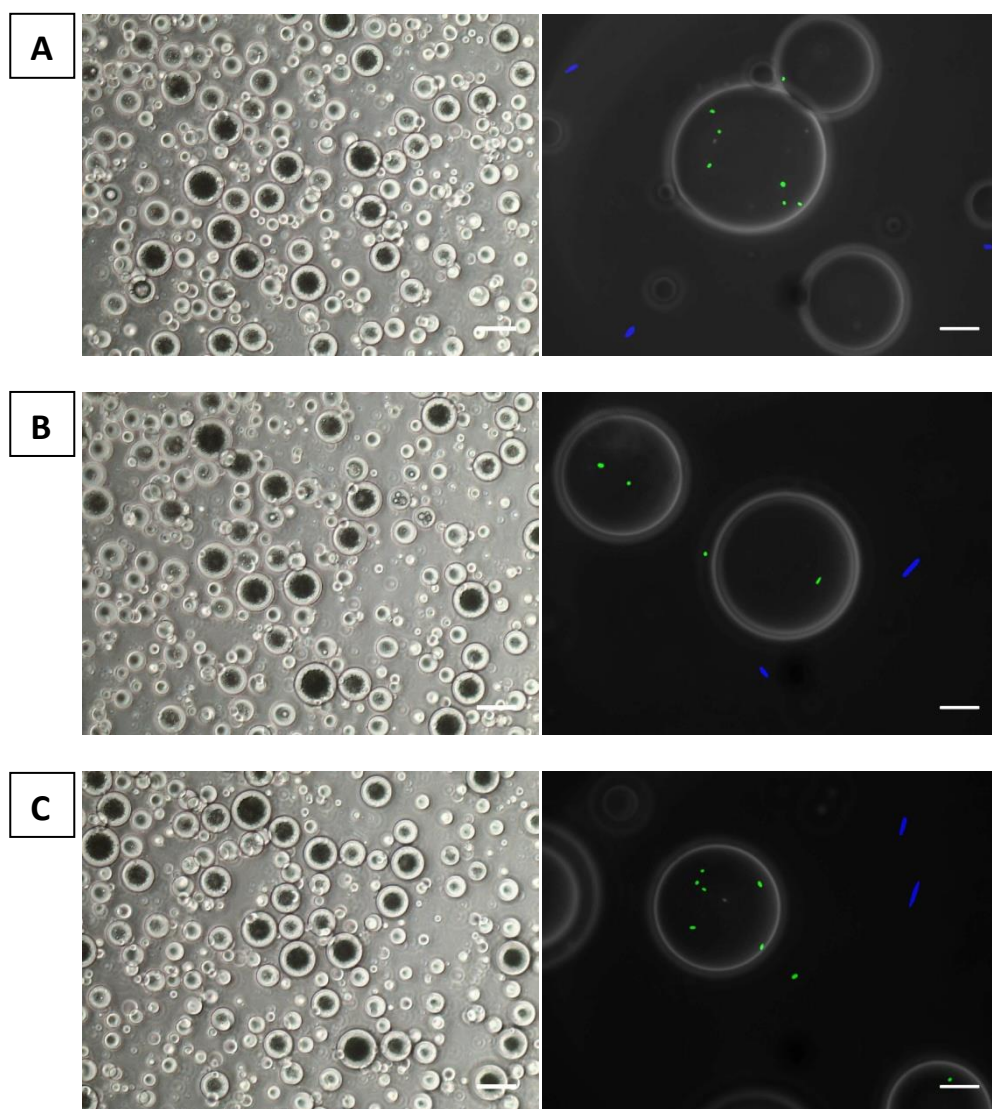


Figure 4.8 Micrographs of $W_1/O/W_2$ double emulsions using optical (left) and fluorescence (right) microscopy at 0, 3 and 7 days (A, B, and C respectively). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 20% containing *E. coli*-GFP in the inner W_1 phase and *L. acidophilus* in the outer W_2 phase and stabilised with 5% Tween80 in W_2 and 2% PGPR in the oil phase. Scale bar: 50µm (left) and 10µm (right).

4.3.7 Effect of encapsulation and storage on *E. coli* viability in W₁/O/W₂ emulsions

To understand how *E. coli* cells are affected by encapsulation and storage in W₁/O/W₂ emulsions the viability of bacteria by culture on media was measured. There was no significant difference in reduction in log CFU/ml of *E. coli* cells in W₁/O/W₂ emulsions compared to unencapsulated (control) over 7 days of storage (Figure 4.9).

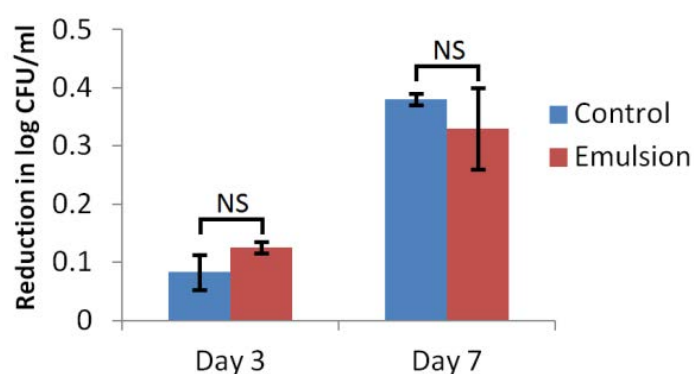


Figure 4.9 Viability of *E. coli*-GFP in W₁/O/W₂ emulsion after 3 and 7 days. The reduction in log number of *E. coli*-GFP cells in serum phase was quantified by plate counting after 3 and 7 days relative to day 0. Control was made with de-ionised water inoculated with *E. coli*-GFP. The W₁/O/W₂ emulsion was made with 20% W₁ and stabilised with 5% Tween80 in W₂ and 2% PGPR in the oil phase. Results are taken from a minimum of 3 independent experiments. Bars represent mean \pm SEM taken from a minimum of 3 independent experiments. The data was analysed with Student's *t*-Test.

4.4 Discussion

The stability of the primary W₁/O emulsion at different concentrations of *E. coli*-GFP cells was studied with varying PGPR and W₁ concentration. The primary W₁/O emulsion did not form at high concentration of *E. coli*-GFP cells (10⁹ CFU/ml) when the concentration of W₁ was high (40%) and PGPR was low (1%). Moreover, the same also occurred when the cells were dead showing that the disruption of W₁/O

emulsion formation was not due to any metabolic activity of the cells. It was also observed that when the W_1/O emulsions were not able to form, multiple $W/O/W/O$ emulsion morphology appeared. This type of emulsion was described as a super multiple emulsion (Galindo-Alvarez *et al.*, 2011) and appeared as part of the emulsion morphology evolution during catastrophic phase inversion which occurs when one of formulation variables (e.g. surfactant affinity) or composition variables (e.g. oil/water ratio) is changed during the homogenising process (Galindo-Alvarez *et al.*, 2011). This might be due to the lipophilic nature of PGPR allowing it to adsorb to the bacterial lipid membrane reducing its concentration within the oil phase. However, more investigation is required to understand how the presence of *E. coli*-GFP could lead to a multiple emulsion morphology formation.

The stability of the $W_1/O/W_2$ emulsion was investigated in the presence of *E. coli*-GFP (in W_1) and/or *L. acidophilus* (in W_2) during 7-day storage. During storage for 7 days there were no significant changes in D (4, 3) or creaming stability with the presence of *E. coli*-GFP and/or *L. acidophilus* within the $W_1/O/W_2$ emulsions. Furthermore, microscopic observation showed no alteration to the $W_1/O/W_2$ emulsion microstructure in the presence of bacteria. However, the encapsulation efficiency was significantly ($P<0.05$) reduced after 3 and 7-day storage. Moreover, some *E. coli*-GFP cells were observed in the W_2 phase after 3 and 7 days.

Increasing the concentration of lipophilic surfactant in the oil phase can increase the stability and encapsulation efficiency of $W_1/O/W_2$ emulsions. Hattrem *et al.* (2014) investigated the stability and encapsulation efficiency of tartrazine in $W_1/O/W_2$ emulsions using different concentrations of PGPR (0.4, 0.8, 1.2, 1.6 and 2%) over 90-day storage. They found that the stability of emulsions was increased at the higher concentrations of PGPR and the encapsulation efficiency of tartrazine in

$W_1/O/W_2$ emulsions stabilised with 2% PGPR was more than 95% while with 0.4% PGPR it was 60%. In another study magnesium was encapsulated in the inner W_1 phase of $W_1/O/W_2$ emulsion and stability was assessed by measuring globule size and encapsulation efficiency at two storage temperatures (4 and 25°C) over 30 days (Bonnet *et al.*, 2009). At both temperatures, the stability and encapsulation efficiency was higher with increasing amounts of PGPR and with high viscosity oils (Bonnet *et al.*, 2009). In another study, the encapsulation efficiency of resveratrol was about 20% higher after 30-day storage when the concentration of PGPR was increased from 5% to 30% (Matos *et al.*, 2014). However, there is a limited acceptability of Tween80 and PGPR surfactants in the food industry (Norn, 2015). Therefore, the addition of other natural substances to diminish or replace the quantity of these surfactants in food is desirable (Norton and Norton, 2010).

The addition of proteins can increase the stability and encapsulation efficiency of $W_1/O/W_2$ emulsions. When faba bean protein was used to replace Span80 (lipophilic surfactant) in the W_1 phase the $W_1/O/W_2$ emulsion showed better stability and encapsulation efficiency over 7-day storage (Koberstein-Hajda and Dickinson, 1996). The addition of sodium caseinate to W_2 in addition to the presence of PGPR in the oil phase enhanced the stability of $W_1/O/W_2$ emulsion containing vitamin B₁₂ in W_1 which exhibited encapsulation efficiency greater than 96% (Giroux *et al.*, 2013). Compared to sodium caseinate, the addition of sodium caseinate–dextran conjugate to W_2 in the presence of PGPR in the oil phase increased the encapsulation efficiency of vitamin B₁₂ in $W_1/O/W_2$ emulsion from 70% to 80% (Fechner *et al.*, 2007). The inclusion of BSA in the W_1 or W_2 phases of the $W_1/O/W_2$ emulsion stabilised with Span80 increased the encapsulation efficiency of NaCl (Garti *et al.*,

1994). The authors suggested that BSA formed a barrier at the W_1/O or O/W_2 interfaces preventing the migration of NaCl from W_1 to W_2 .

Natural polymers have been used to increase the stability and encapsulation efficiency of $W_1/O/W_2$ emulsions. In one study $W_1/O/W_2$ emulsions stabilised with PGPR showed increased encapsulation efficiency of NaCl after one-month storage at chilling temperature when gelatine was present in W_1 (Sapei *et al.*, 2012). The presence of betaine in W_2 increased the stability and encapsulation efficiency of $W_1/O/W_2$ emulsions stabilised with PGPR (Kanouni *et al.*, 2002). In a more recent study by Zhang and Reineccius (2016) showed that gum arabic in W_2 in the presence of PGPR in the oil phase could stabilise $W_1/O/W_2$ emulsions and compared to Tween20 and modified starch provides around 35% higher encapsulation efficiency of a marker dye.

The pressure difference between the inside and outside of the globule is known as Laplace curvature pressure and it works against the stability of the emulsion. Stable $W_1/O/W_2$ emulsions require a balance between the Laplace and osmotic pressures (Kanouni *et al.*, 2002). The addition of a small quantity of electrolytes in W_1 allows counteracting the Laplace pressure effect, however if the concentration of electrolytes is too high the globules will swell and destabilise. Matos *et al.* (2013) showed that after 21 days the encapsulation efficiency of a marker dye in $W_1/O/W_2$ emulsion was 95.2% with 0.1 NaCl in W_1 compared to 91.1% with 0.2 M NaCl in W_1 (Matos *et al.*, 2013).

In this study, the viability of *E. coli*-GFP in W_1 was not affected by encapsulation and during storage in $W_1/O/W_2$ emulsion after 7 days. Using microscopy, it was observed that *L. acidophilus* had an elongated morphology after 3 and 7 days. Masaki *et al.*

(1990) studied effect of Tween80 on *Mycobacterium avium* and found that Tween80 possesses bacteriostatic activity which resulted in cell elongation. Since *L. acidophilus* cells were in W_2 phase, their change in morphology could probably be due to their interaction with Tween80. It was also found that bacteria in W_1 and W_2 phase were present as planktonic forms during the incubation period. The growth of bacteria can be affected by space limitation within the emulsion. In previous studies, it was shown that due to space limitation bacteria in O/W emulsion formed colonies and as opposed to growing in planktonic form and this was more evident when the oil phase was increased (Parker *et al.*, 1995) and globule size was decreased (Brocklehurst *et al.*, 1995). The effect of the size confinement of the colloidosomes hindered glucose metabolism restricting the growth and reproduction of *Lactobacillus crispatus* (Keen *et al.*, 2012). On the other hand, Boedicker *et al.* (2009) showed that when confined in a small volume *P. aureginosa* initiate QS which is necessary for growth, biofilm formation, motility and other critical functions. The authors visualized QS within droplets using a GFP reporter that was expressed in *P. aureginosa* because of the accumulation of autoinducers (signalling molecules). However, in our study the inner W_1 and outer W_2 phases of the $W_1/O/W_2$ emulsions lacked nutrients and therefore they were unable to grow. Further, studies are required to investigate whether space limitation does affect bacterial growth in $W_1/O/W_2$ emulsions.

The migration of solutes between the phases may occur due to diffusion as well as globule rupture or erosion (Ficheux *et al.*, 1998). The dominating release mechanism of anthocyanins from $W_1/O/W_2$ emulsions was shown to be via the coalescence of W_1 droplets with the W_2 phase (Frank *et al.*, 2012). In another study, the release of NaCl from $W_1/O/W_2$ emulsions was governed by diffusion and not by coalescence of W_1 droplets with W_2 or globule rupture (Sapei *et al.*, 2012). Since bacteria can be

considered as particles it is more likely that the latter two mechanisms are responsible for their movement towards the W_2 phase. Further work is needed to investigate the mechanism by which *E. coli*-GFP moves from the W_1 to W_2 phase.

4.5 Conclusion

In this study, the effect of two bacterial species on the stability of $W_1/O/W_2$ emulsions was investigated for the first time. The presence of *E. coli*-GFP affected the emulsion structure during the first step of the emulsification process, but had no effect on emulsion structure during storage. The presence of *E. coli*-GFP and/or *L. acidophilus* had no effect on the stability of the $W_1/O/W_2$ emulsions after 7 days of storage. Also, the initial encapsulation efficiency was more than 99% immediately after emulsification and the encapsulation stability was significantly decreased after 3 and 7 days but remained over 92%. Furthermore, some *E. coli*-GFP cells were observed in W_2 after 3 and 7 days of storage. Further work is needed to improve the stability of the system to maintain high encapsulation efficiency. It was also shown that the viability of *E. coli*-GFP was not affected by their presence in the W_1 phase of the $W_1/O/W_2$ emulsion. These findings are significant for industry showing that $W_1/O/W_2$ emulsions have the potential to be used for microbiological applications. However, further work is required to understand the mechanism by which bacteria migrate from W_1 to W_2 during storage and this will enable formulating $W_1/O/W_2$ emulsions that can either maintain high encapsulation efficiency or upon trigger can destabilise and release bacteria at the desired time.

CHAPTER 5: UNDERSTANDING AND CONTROLLING THE MECHANISM OF *ESCHERICHIA COLI* RELEASE IN DOUBLE $W_1/O/W_2$ EMULSION GLOBULES UNDER HYPER-OSMOTIC PRESSURE

5.1 Introduction

There is a lack of understanding of the mechanism by which bacteria are released from the W_1 phase of $W_1/O/W_2$ emulsions. In the case of chemical substances release was found to vary between diffusion-dependent transfer through the oil phase (Yanagie *et al.*, 2011; Sapei *et al.*, 2012) and/or oil globule bursting (Cárdenas and Castro, 2003).

Bacteria can either destabilise (Ly *et al.*, 2006; Ly *et al.*, 2008) or stabilise (Dorobantu *et al.*, 2004); Firoozmand and Rousseau, 2014) the structure of a single emulsion. *E. coli* can metabolise glucose and grow within water droplets of W/O emulsions and excrete products that diffuse through the oil phase and into surrounding water droplets creating an osmotic mismatch leading to water flux and droplet shrinkage at rates depending on the strain, species, and number of the microbial cells (Chang *et al.*, 2015b). However, studies investigating the effect of bacteria on stability of $W_1/O/W_2$ emulsions and the effect of $W_1/O/W_2$ emulsion structure on bacterial growth and viability are lacking.

$W_1/O/W_2$ emulsions are kinetically unstable due to the presence of two thermodynamically unstable interfaces (McClements *et al.*, 2009) and their stability requires a balance between the Laplace and osmotic pressures (Kanouni *et al.*, 2002; Mezzenga *et al.*, 2004). By destabilising their structure, release of hydrophilic compounds is possible, and this can either be thermally-induced (Rojas and Papadopoulos, 2007; Rojas *et al.*, 2008) or osmotically-induced (Cárdenas and

Castro, 2003). The flux of water from W_1 to W_2 due to a higher concentration gradient in W_2 could destabilise the $W_1/O/W_2$ emulsions as the globules shrink and/or collapse (Jiao *et al.*, 2002; Mezzenga *et al.*, 2004). However, such destabilisation might be desired in certain applications to control release of substances from the W_1 phase. This instability, if controlled could allow for the release of entrapped hydrophilic substances at the proper time. Thus, oil globules should be stable enough to contain the materials entrapped within their inner W_1 phase but at the same time susceptible to physicochemical changes so that they breakdown to deliver the entrapped hydrophilic substances to the outer W_2 phase (Cárdenas and Castro, 2003).

Release of substances from W_1 to W_2 in $W_1/O/W_2$ emulsions can occur by two general mechanisms; breaking of the oil globules or transport through the oil phase without breaking of the oil globules. Many studies investigated the release of compounds from $W_1/O/W_2$ emulsion globules such as NaCl (Pays *et al.*, 2002), drugs (Yanagie *et al.*, 2011) and hormones (Sato *et al.*, 1995). Several mechanisms have been suggested for the movement of solutes or compounds in between the W_1 and W_2 phases and these include surfactant-facilitated transport through reverse micelles (Omotosho *et al.*, 1986), hydrated surfactant and/or diffusion through thin surfactant lamellae (Colinart *et al.*, 1984). Tedajo *et al.* (2005) has shown that by diluting the $W_1/O/W_2$ emulsion in a hypo-osmotic solution oil globule bursting occurs leading to the release of antiseptics. In another study, it was reported that NaCl released into W_2 due to oil globule bursting was shown to increase with $W_1/O/W_2$ emulsion formulations containing higher amounts of W_1 (Cárdenas and Castro, 2003). However, these studies investigated the release mechanism by oil globule

bursting due to swelling which occurs when the osmotic pressure in W_2 is higher than W_1 created by the presence of NaCl in W_2 .

The stability and release properties of $W_1/O/W_2$ emulsions can be controlled by altering the oil/water ratio, osmotic balance between the two aqueous phases, and the type and/or concentration of the emulsifiers (Cárdenas and Castro, 2003). However, no study has yet investigated how insoluble particles such as bacteria can be delivered from the inner to the outer phase of $W_1/O/W_2$ emulsions. In this chapter, the mechanism by which release of bacteria occurs was microscopically visualised and the effect of the structure of $W_1/O/W_2$ emulsions on bacterial release facilitated by the presence of NaCl in W_2 was investigated. Also, the amount of inner W_1 phase and hydrophilic surfactant in W_2 were changed and their effect on $W_1/O/W_2$ emulsion stability in the presence of GFP-tagged *E. coli* was analysed. Furthermore, the effects of encapsulation, release, and chemistry of emulsion on *E. coli* was investigated.

5.2 Materials and Methods

5.2.1 Materials

Includes all the materials in Chapter 4, Section 4.2.1, with the addition of the following:

- PI was purchased from Sigma-Aldrich (United Kingdom)
- NaCl 99% was purchased from Sigma-Aldrich (Germany).

5.2.2 Microbial cultures

E. coli strain K-12, MG1655 (ATCC 47076) and its derivative *E. coli* SCC1 (MG1655 $P_{A1/04/03}$ -*gfpmut3*^{*}, expressing GFP from the chromosome and subsequently referred

to as *E. coli*-GFP) (Miao *et al.*, 2009) were maintained on tryptic soy agar petri dishes at 4°C. Cells were transferred into 50 ml of tryptic soy broth in Erlenmeyer flask (250 ml), incubated at 37°C for 24 hours shaking at 150 rpm and sub-cultured to 50 ml of tryptic soy broth in Erlenmeyer flask (250 ml) for a further 2 hours. For obtaining cells in the exponential phase cells were harvested by centrifugation (10,000 g, 10 minutes) using 15 ml Falcon tubes and washed in *PBS* (phosphate buffered saline) buffer solution twice.

5.2.3 Rheological measurements

Rheological characterisation of emulsion separate components was done by measuring the viscosity of the oil phase without or with varying concentrations of PGPR (2, 4, 6 or 8%), W_2 phase without or with varying concentrations of Tween80 (0.5, 1, 5 or 10%) and W_1/O emulsion with different W_1 volume fraction (20 or 40% W_1) in the presence of *E. coli*-GFP was performed at 25°C using AR-G2 rheometer (TA instruments, New Castle, Delaware USA) on a parallel plate geometry (diameter 40mm). The apparent viscosity was measured over a shear rate range 0.1-100 s⁻¹. Briefly, 1 mL of sample was placed between the cone and the plate, and measurement was started immediately. In total, 30 data points were recorded at 10 s intervals during the shearing. Shear stress was determined as a function of shear rate. Data was fitted to Power-law model (Barnes *et al.* 1989):

$$\eta = K \cdot \dot{\gamma}^{n-1} \quad (5.1)$$

where; η refers to viscosity (Pa.s), K to consistency coefficient (Pa.sⁿ), $\dot{\gamma}$ to shear rate (s⁻¹), and n to flow behaviour index (dimensionless).

5.2.4 Preparation of $W_1/O/W_2$ emulsions

Double $W_1/O/W_2$ emulsions were prepared using a high shear mixer homogeniser (Silverson L5M) at room temperature using a two-step emulsification process (Figure A6). In the first step, primary W_1/O emulsions were made. An oil phase was prepared by dissolving 2 wt% PGPR in sunflower oil. The inner aqueous phase (W_1) consisting of de-ionised water was emulsified (120 seconds) into the oil phase ($W_1:O$ phase ratio of 20:80 or 40:60). For microbial-encapsulation de-ionised water containing the washed bacteria (2×10^8 CFU/ml) was used as W_1 . In the second step $W_1/O/W_2$ emulsion was made. The outer aqueous phase (W_2) was prepared by dissolving Tween80 (0.5, 1, 5 or 10 wt%) in de-ionised water at 60°C for approximately 15 minutes. The previously prepared primary W_1/O emulsion was emulsified (60 seconds) into W_2 to form the $W_1/O/W_2$ emulsion ($W_1/O:W_2$ ratio of 20:80). The rotational speeds for homogenising the different formulations of primary W_1/O and $W_1/O/W_2$ emulsions are shown in Table A1. Finally, 20 ml of the double $W_1/O/W_2$ emulsion was replaced by either de-ionised water or NaCl solution (final concentration of 0.02 M, 0.04 M, 0.085 M or 0.17 M) to alter the osmotic balance and the whole 100 ml of $W_1/O/W_2$ emulsion was transferred into sterile Erlenmeyer flask (500 ml) and incubated at 25°C for 1 or 6-hour period shaking at 100 rpm to ensure homogeneous mixing.

5.2.5 Microscopic observation of the $W_1/O/W_2$ emulsions

Video microscopy was used to observe the release mechanism of *E. coli*-GFP cells from $W_1/O/W_2$ emulsion globules. Fluorescent and optical microscopy was used to monitor *E. coli*-GFP cells in $W_1/O/W_2$ emulsions during incubation.

5.2.5.1 Video time-lapse for observation of oil globule bursting and tracking of *E. coli*-GFP

For video microscopy, the sample was placed on a microscope slide and the video was recorded under objective lens 40x magnification with a Moticam 10 camera via Motic Images Plus video acquisition software at 17fps. The light source used to excite the GFP was a mercury arc lamp and the emission was observed at 509 nm.

5.2.5.2 Fluorescent and optical imaging of the $W_1/O/W_2$ emulsions and *E. coli*-GFP

This procedure was done as in Section 4.2.4.

5.2.6 Measuring encapsulation efficiency (%) and quantifying the release of *E. coli*-GFP during emulsification and storage

Due to the differences in density between the W_2 phase and the oil globules creaming or phase separation occurs when $W_1/O/W_2$ emulsion is un-mixed and it partitions into a serum phase (W_2 phase) and a cream layer (containing oil globules) (Robins *et al.*, 2002). The number of *E. coli* cells in the serum phase was measured immediately after preparation and as a function of storage time. Five millilitre sample of $W_1/O/W_2$ emulsion was collected and the serum phase was removed using a 5ml graduated syringe. *E. coli* cell counts were made using serial dilutions in PBS (phosphate buffered saline) buffer solution and plating on tryptic soy agar using the Miles & Misra technique (Miles *et al.*, 1938). Colony forming units per millilitre (CFU/ml) were then calculated. As the serum phase contains no oil globules only unencapsulated (or those released when oil globules burst) viable bacteria could grow to colony forming units which could be counted. The encapsulation efficiency and release of *E. coli*-GFP were calculated by the following equations:

$$\text{Encapsulation efficiency} = ((N_0 - N) / N_0) \times 100\% \quad (5.2)$$

$$\text{Release} = (\text{Log}_{10} N_T - \text{Log}_{10} N) \quad (5.3)$$

Where N is the number of unencapsulated viable bacterial cells immediately after forming the $W_1/O/W_2$ emulsion, N_0 displays the free viable bacterial cells before encapsulation and N_T displays the unencapsulated viable bacterial cells after incubation period.

5.2.7 Characterisation of $W_1/O/W_2$ emulsion stability

The stability of the $W_1/O/W_2$ emulsions was monitored by measuring the oil globule size [D (4, 3)] and creaming thickness during incubation.

5.2.7.1 Measurement of oil globule size of double $W_1/O/W_2$ emulsions

The particle size distribution of oil globules in the $W_1/O/W_2$ emulsion was measured immediately after preparation and as a function of storage time using a laser diffraction particle size analyser (Malvern Mastersizer 2000, Malvern Instrument Ltd, Worcestershire, UK), equipped with a He-Ne laser ($\lambda = 633 \text{ nm}$). This procedure was done as in Section 3.2.6.1.

5.2.7.2 Observation of phase separation

The cream height fraction of the $W_1/O/W_2$ emulsion was measured immediately after preparation and as a function of storage time. This procedure was done as in Section 3.2.6.2.

5.2.8 Determining the viability and health of *E. coli* (MG1655) after encapsulation and release using epifluorescence microscopy

E. coli MG1655 in serum phase or de-ionised water (control) was stained by adding DAPI (4µl/ml) and PI (4µl/ml) and incubated in the dark for 30 minutes. A nucleopore black polycarbonate membrane filter with 0.2 µm pore size and 13 mm diameter was mounted shiny side, uppermost on a membrane Swinnex filter holder (Millipore) and the stained solution was passed through the membrane filter by injecting slowly with a 5ml graduated syringe. The membrane filter was placed shiny side uppermost on a glass slide and a drop of immersion oil added to its surface and gently covered with a cover slip. The cells were then counted under objective lens of 100x magnification with a fluorescent microscope (Zeiss Axiolab) equipped with a mercury arc lamp and the emission was observed at 461 nm (DAPI) and 645 nm (PI). For each filter, ten microscopic (0.1 mm x 0.1 mm) fields were randomly selected and all cells within each field were counted and the average number of cells per field was calculated (Hobbie *et al.*, 1977). The serum phase volume was adjusted to yield a minimum of 400 total cell counts in 10 counting fields. The total number of *E. coli* cells from each sample of serum phase was calculated by multiplying the total number of fields with the average number of cells per field.

5.2.9 Statistical analysis

Statistical analysis was done as described in Section 3.2.10 and each experiment was conducted in triplicate (N=3).

5.3 Results

5.3.1 Primary W_1/O emulsion characterization

The stability of $W_1/O/W_2$ emulsions can be influenced by the size of W_1 droplets (Weiss and Muschiolik, 2007). In this study, the homogenisation conditions were as such that the average mean size distribution of W_1 droplets was comparable (3-4 μm ; Appendix, Figure A7 and A8) in all primary W_1/O formulations.

5.3.2 Rheological characterisation of the primary W_1/O emulsion and phases

To understand how the concentration of W_1 and the concentration of surfactants affect the rheological properties of the $W_1/O/W_2$ emulsion, the viscosity of the oil phase, W_2 phase and the primary W_1/O emulsion were measured (Table 5.1). The Power-Law model was used to describe the flow curves of the koji and brine mixtures. The rheological parameters of this model are presented Table 5.1. All the samples exhibited non-Newtonian behaviour at shear rates ranging between 0.1 and 100 s^{-1} at 25°C since all the samples had a flow index n of less than 1 (shear thinning). The viscosity of the oil phase was significantly ($P < 0.05$) higher with increasing concentrations of PGPR. There was no significant difference in the viscosity between the W_2 with varying concentrations of Tween80. The viscosity of the W_1/O emulsion was significantly ($P < 0.05$) higher at 40% W_1 compared to 20% W_1 . These results suggest that the surfactant concentration affects the viscosity of the oil phase but not the W_2 phase while the viscosity of the primary W_1/O emulsion is affected by the amount of W_1 .

Table 5.1 Viscosity (mPas.s) measurements of the oil phase, W_2 phase and primary W_1/O emulsion. The oil phase was prepared with sunflower oil without or with PGPR (2%, 4%, 6%, or 8%). The W_2 phase was prepared without or with Tween80 (0.5%, 1%, 5% or 10%). Primary W_1/O emulsions were prepared with W_1 volume percentage of 20% or 40% in the presence of *E. coli*-GFP with 0.085 M NaCl in the W_1 phase and stabilised and 2% PGPR in the oil phase. Results are taken from a minimum of 3 independent experiments. The apparent viscosity (mPas.s), consistency constant K and power-law index n for each sample was measured over a shear rate range $0.1\text{-}100\text{ s}^{-1}$ at 25°C . The viscosity was taken as the average of the final 7 points where the curve becomes a plateau.

Component	Viscosity (mPa.s)	K	n
Oil phase			
Sunflower	14.21 ± 0.89^a	0.04	0.78
Sunflower oil + 2% PGPR	22.73 ± 4.05^a	0.04	0.87
Sunflower oil + 4% PGPR	22.05 ± 0.90^{ab}	0.04	0.86
Sunflower oil + 6% PGPR	34.53 ± 6.29^{bc}	0.05	0.91
Sunflower oil + 8% PGPR	44.58 ± 3.92^c	0.06	0.93
W_2 phase			
Water	0.83 ± 0.47^a	0.03	0.83
Water + 0.5% Tween80	0.44 ± 0.52^a	0.03	0.04
Water + 1% Tween80	0.47 ± 0.01^a	0.04	0.01
Water + 5% Tween80	0.54 ± 0.01^a	0.04	0.05
Water + 10% Tween80	0.94 ± 0.07^a	0.02	0.27
Primary W_1/O emulsion with <i>E. coli</i>-GFP in W_1			
W_1/O (20:80) + 2% PGPR	20.96 ± 1.66^a	0.04	0.84
W_1/O (40:60) + 2% PGPR	55.26 ± 8.59^b	0.08	0.91

The data was analysed with one-way ANOVA and Student's *t*-Test

a, b, c means \pm standard deviation with different letters are significantly different

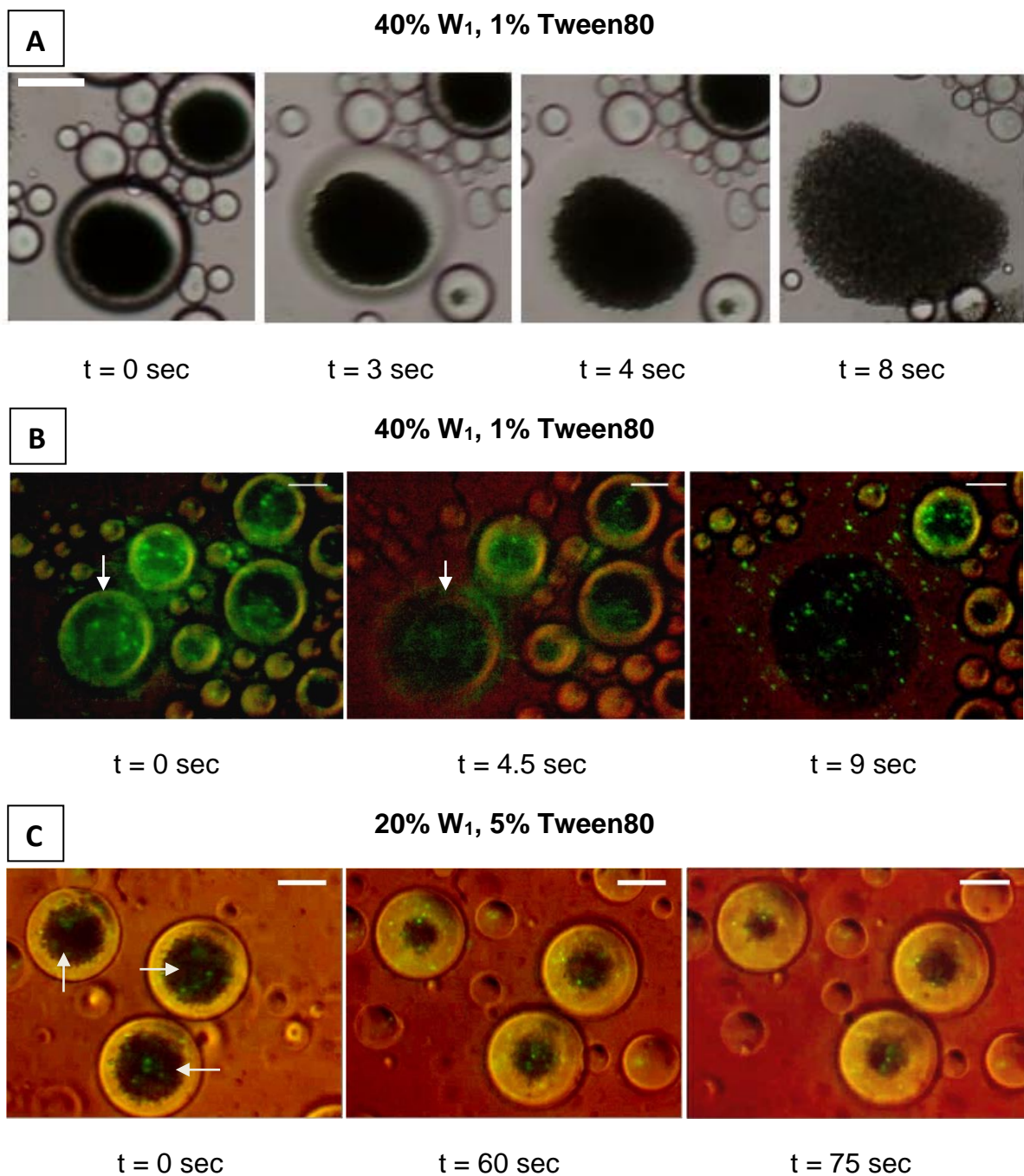
5.3.3 Encapsulation efficiency (%) of *E. coli*-GFP cells in $W_1/O/W_2$ emulsions

The bacterial counts for *E. coli*-GFP before and after the encapsulation process were used to calculate the encapsulation efficiency from different $W_1/O/W_2$ emulsions. The results indicated successful entrapment of viable bacterial cells (>99.8%) in all the prepared $W_1/O/W_2$ emulsions (Appendix, Table A2).

5.3.4 Microscopic observation of oil globule bursting and *E. coli*-GFP release

Using video-microscopy the bursting mechanism responsible for the release of *E. coli*-GFP from the oil globules after adding salt in W_2 was observed. Moreover, the oil globule bursting phenomenon was a reproducible observation and occurred for the majority of the oil globules. For all the double emulsion formulations when no NaCl was added to the W_2 phase the oil globules did not burst and the W_1 droplets encapsulating *E. coli*-GFP remained within the oil globules. Figures 5.1A and 5.1B show snapshots taken after adding NaCl to W_2 of $W_1/O/W_2$ emulsion made with 40% W_1 and 1% Tween80 (Appendix, Video A1 and A2 respectively). The membrane of the oil globule ruptures and W_1 droplets encapsulating *E. coli*-GFP cells are released into W_2 . Figure 5.1C shows snapshots taken after adding NaCl to W_2 of $W_1/O/W_2$ emulsion made with 20% W_1 and 5% Tween80 (Appendix, Video A3). There was no oil globule bursting observed, however, loss of the W_1 droplets was evident while *E. coli*-GFP cells remained within the oil phase of the oil globules. Figure 5.1D shows snapshots of a coalescence event between two oil globules after adding NaCl to W_2

of $W_1/O/W_2$ emulsion made with 40% W_1 and 1% Tween80 containing *E. coli*-GFP in W_1 . (Appendix, Video A4). *E. coli*-GFP cells remained within the newly coalesced oil globule. These results suggest that the release of *E. coli*-GFP into W_2 occurs due to the rupturing of the oil globule's interfacial film and seems to be independent of diffusion and coalescence between the oil globules does not result in release of *E. coli*-GFP.



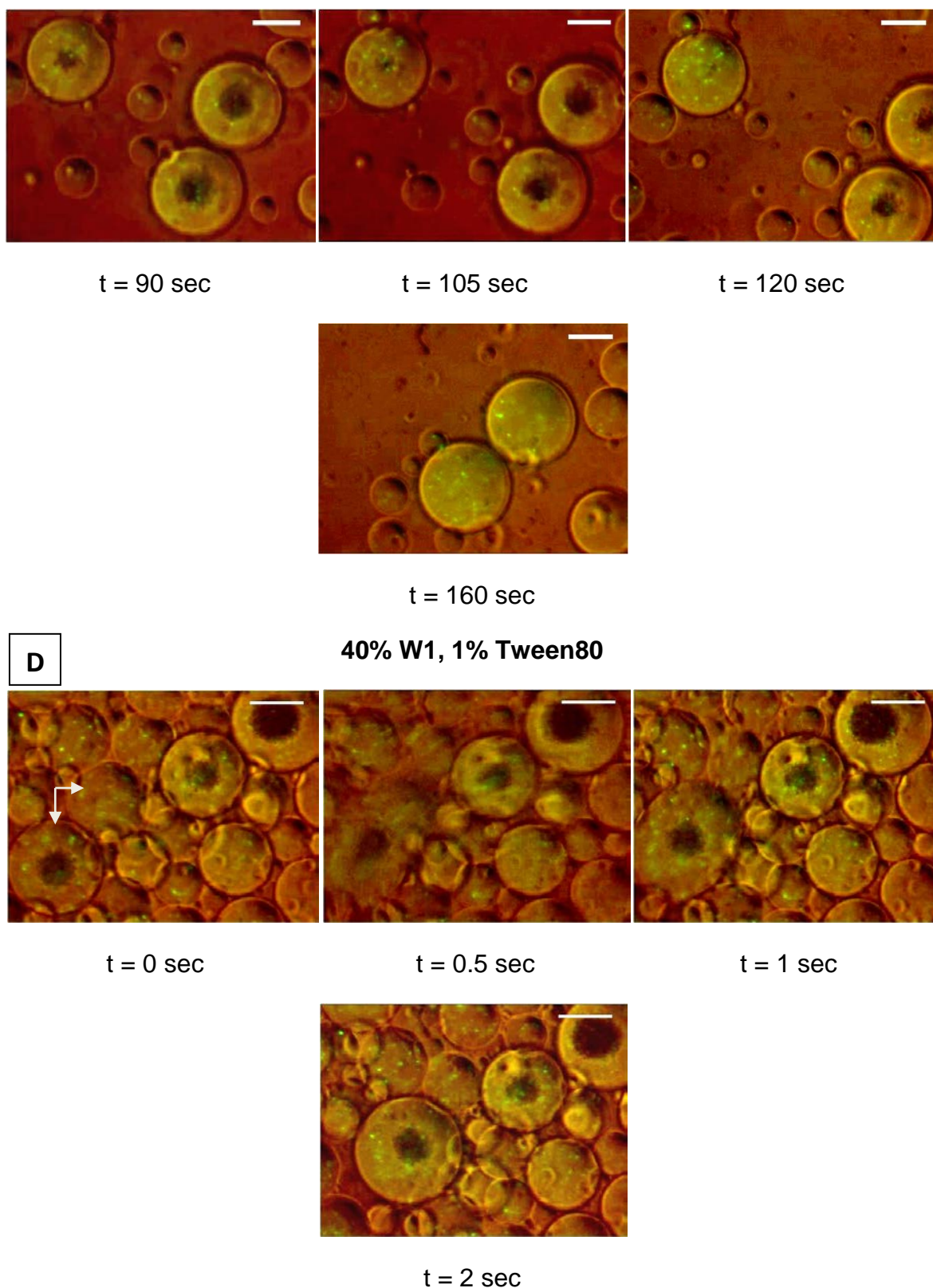


Figure 5.1 Optical and fluorescence snap shot images obtained from video-microscopy showing a scenario with oil globule bursting and release of W_1 droplets (A) or *E. coli*-GFP (B) after adding NaCl in W_2 and another scenario with no oil globule bursting and release of *E. coli*-GFP (C) after adding NaCl in W_2 and coalescence of oil globules with no release of *E. coli*-GFP (D) after adding NaCl in W_2 . A) Optical image of oil globule bursting and release of the W_1 into

W₂ phase. B) Fluorescent images of burst release of *E. coli*-GFP (green) from an oil globule (arrows) in a W₁/O/W₂ emulsion at different time points. C) Fluorescent images of *E. coli*-GFP (green) within the oil globules during the loss of the W₁ droplets due to the presence of NaCl in the W₂ phase. The inner W₁ droplets can be observed as a dark mass within the oil globules (arrows). D) Fluorescent images of *E. coli*-GFP (green) within two oil globules (arrows) that are undergoing coalescence and during the loss of the W₁ droplets due to the presence of NaCl in the W₂ phase. The W₁/O/W₂ emulsion was prepared with 40% W₁ containing bacteria and stabilised with 1% Tween80 containing 0.085 M NaCl in the W₂ phase (A, B, and D) or 20% W₁ containing bacteria and stabilised with 5% Tween80 containing 0.085 M NaCl in the W₂ phase (C).

5.3.5 Influence of NaCl on release of *E. coli*-GFP

To understand the effect of varying the structure of W₁/O/W₂ emulsion on release of bacteria with and without NaCl in W₂ the release of *E. coli*-GFP over time was quantified (Figures 5.2, 5.3 and 5.4). Also, the W₁/O/W₂ emulsion structure and the localisation of *E. coli*-GFP within the W₁/O/W₂ emulsion were microscopically visualised (Figures 5.5-5.12).

With the formulation containing 40% W₁ and stabilised with 1% Tween80 (Figure 5.2) the release of *E. coli*-GFP was significantly ($P < 0.05$) increased with NaCl compared to without NaCl in W₂ but remained significantly similar with increasing NaCl concentrations. This suggests that the release of *E. coli*-GFP was not NaCl concentration dependent.

At 1% Tween80 and regardless of W₁ concentration the release of *E. coli*-GFP was significantly ($P < 0.05$) higher with NaCl compared to without NaCl in W₂ (Fig. 5.4) showing that destabilising the emulsion by altering the osmotic balance affects the release of *E. coli*-GFP. During no osmotic balance alteration, the oil globules showed no loss of W₁ droplets (Figures 5.5A, 5.6A, 5.7A and 5.8A) and *E. coli*-GFP cells mostly remained within W₁ droplets of the oil globules (Figures 5.9A, 5.10A, 5.11A and 5.12A). However, after adding NaCl in W₂ the oil globules showed loss of W₁

droplets (Figures 5.5A, 5.6A, 5.7A and 5.8A) and *E. coli*-GFP cells were present in the oil phase of the oil globules (Figures 5.10B and 5.11B) and in the W_2 phase (Figures 5.9B and 5.12B). After adding NaCl to W_2 the release of *E. coli*-GFP was significantly ($P<0.05$) higher at low concentrations of Tween80 (0.5% and 1%) compared to high concentrations of Tween80 (5% and 10%) (Figures 5.3 and 5.4). Interestingly, there was no significant difference in release of *E. coli*-GFP at 20% W_1 and 5% Tween80 with NaCl compared to without NaCl in W_2 (Figure 5.4) whilst the oil globules showed loss of W_1 droplets (Figure 5.6B) and *E. coli*-GFP cells were present mainly in the oil phase of the oil globules (Figure 5.10B). This suggests that the release of *E. coli*-GFP is affected by the concentration of Tween80 during osmotic balance alteration.

After adding NaCl in W_2 the release of *E. coli*-GFP was significantly ($P<0.05$) higher at 40% W_1 when the emulsion was stabilised with 1% Tween80 compared to 5% Tween80 after 6 hours (Figure 5.4). After adding NaCl in W_2 the release of *E. coli*-GFP was significantly ($P<0.05$) higher at 1% Tween80 when W_1 was 40% compared to 20% W_1 after 2 hours and then became non-significant after 4 and 6 hours (Figure 5.4). These results indicate that the amount of W_1 affects the release of *E. coli*-GFP during osmotic balance alteration.

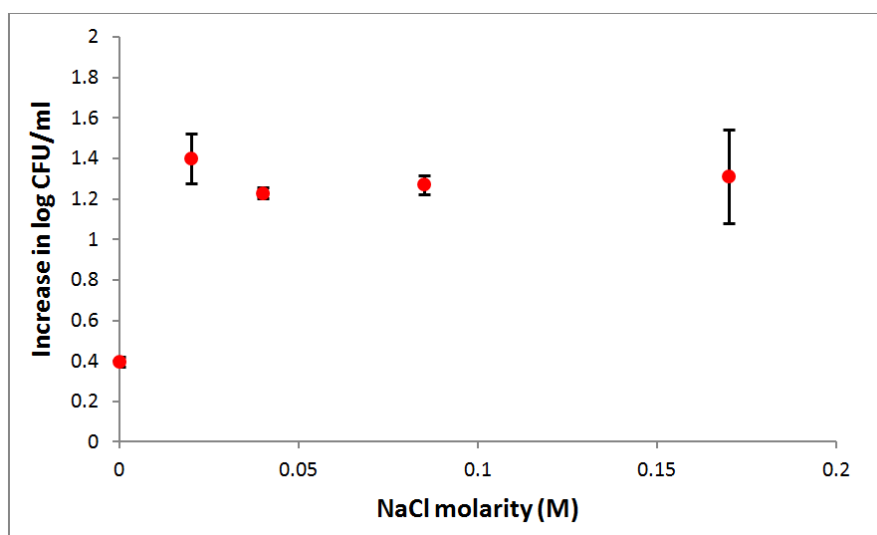


Figure 5.2 Amount of released bacteria in the outer W_2 phase of the $W_1/O/W_2$ emulsions after 1 hour relative to hour 0 incubated at 25°C. The $W_1/O/W_2$ emulsions were prepared with different inner W_1 phase volume percentage of 40% containing *E. coli*-GFP in the W_1 phase and stabilised with 1% Tween80 with or without 0.02 M, 0.04 M, 0.085 M or 0.17 M NaCl in the W_2 phase. Bars represent mean \pm SEM taken from a minimum of 3 independent experiments. The data was analysed with one-way ANOVA.

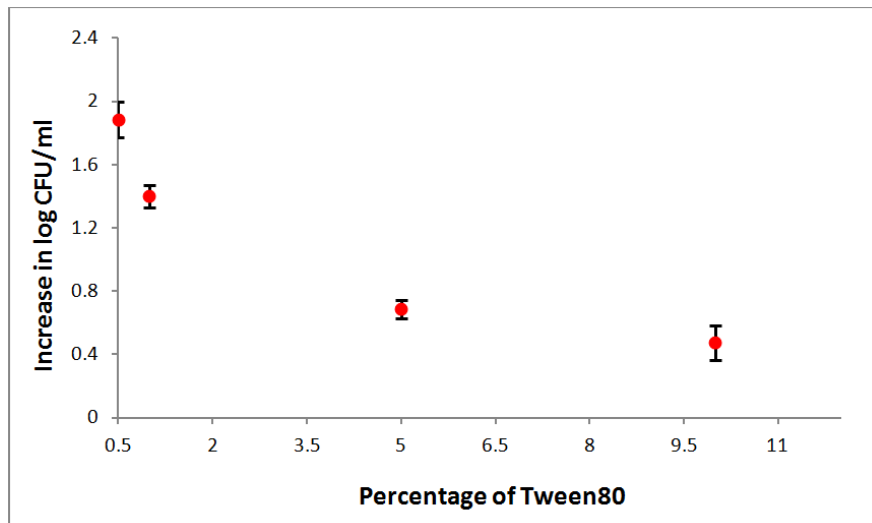


Figure 5.3 Amount of released bacteria in the outer W_2 phase of the $W_1/O/W_2$ emulsions after 1 hour relative to hour 0 incubated at 25°C. The $W_1/O/W_2$ emulsions were prepared with different inner W_1 phase volume percentage of 40% containing *E. coli*-GFP in the W_1 phase and stabilised with 0.5%, 1%, 5% or 10% Tween80 with 0.085 M NaCl in the W_2 phase. Bars represent mean \pm SEM taken from a minimum of 3 independent experiments. The data was analysed with one-way ANOVA.

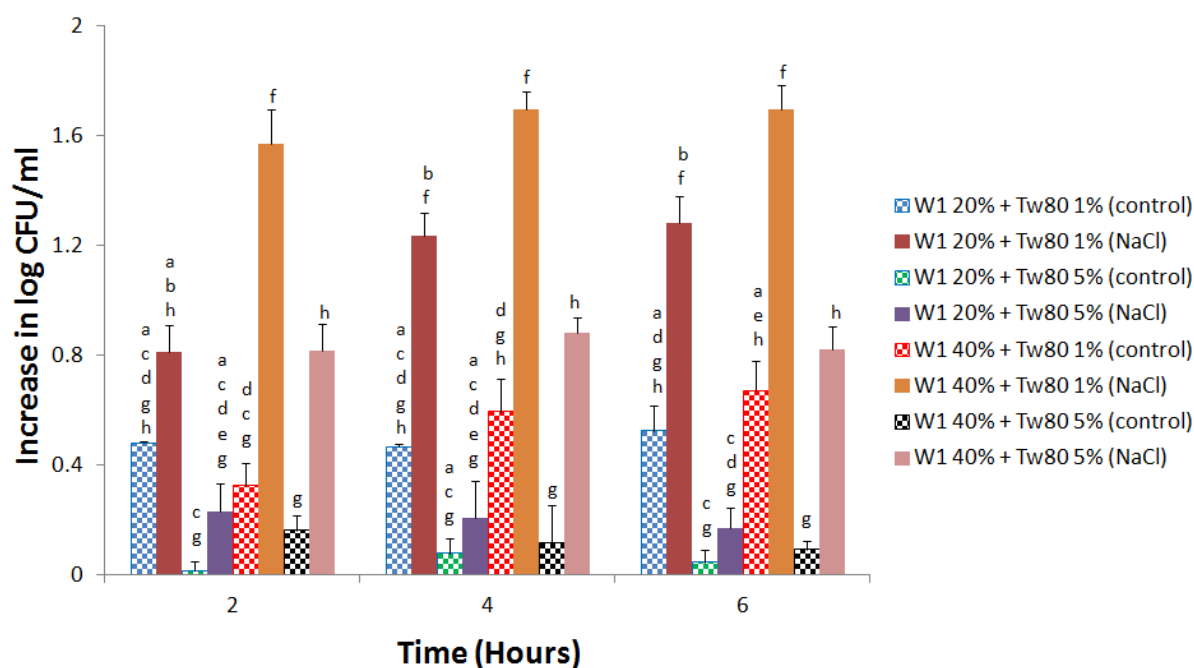


Figure 5.4 Amount of released bacteria in the outer W₂ phase of the W₁/O/W₂ emulsions after 2, 4 and 6 hours incubated at 25°C. The W₁/O/W₂ emulsions were prepared with different inner W₁ phase volume percentage of 20% or 40% containing *E. coli*-GFP in the W₁ phase and stabilised with 1% or 5% Tween80 with or without 0.085 M NaCl in W₂. Bars represent mean ± SEM taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different (P<0.05). The data was analysed with one-way ANOVA.

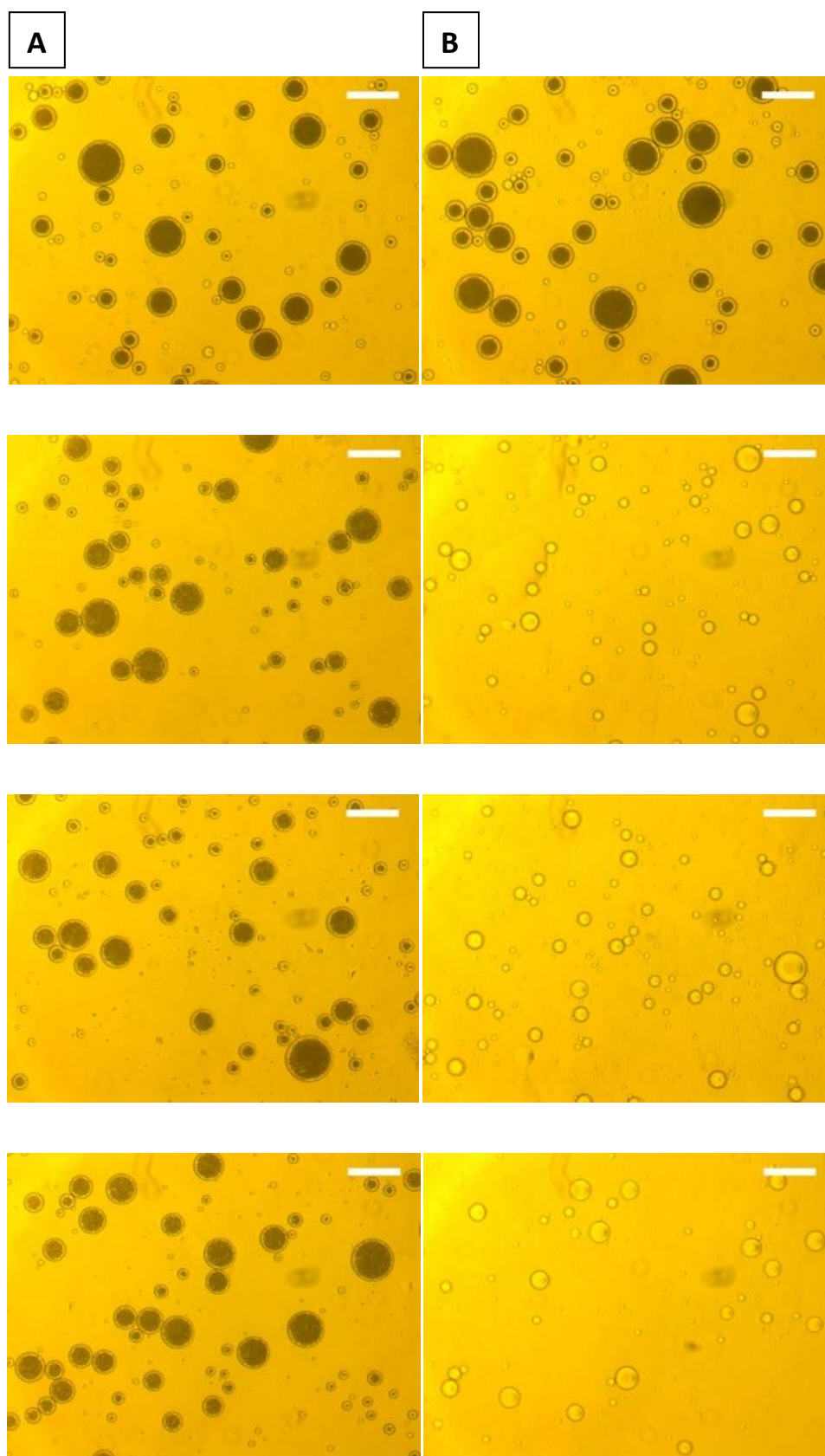


Figure 5.5 Optical microscopy images of $W_1/O/W_2$ emulsions at 0, 2, 4 and 6 hours (from the top). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 20% containing *E. coli*-GFP and stabilised with 1% Tween80 with no (A) or with (B) 0.085 M NaCl in W_2 . Scale bar: 100 μ m.

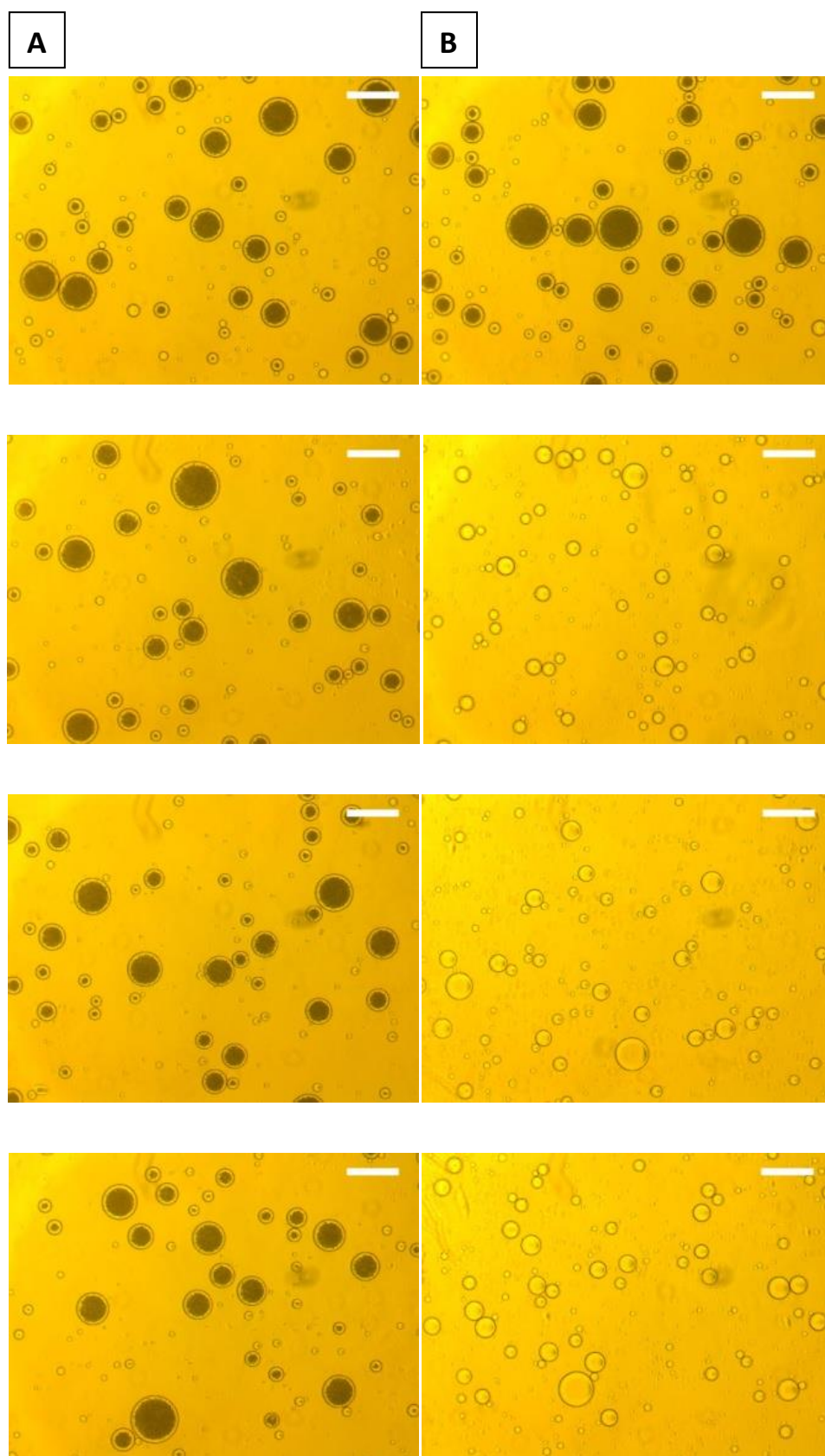


Figure 5.6 Optical microscopy images of $W_1/O/W_2$ emulsions at 0, 2, 4 and 6 hours (from the top). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 20% containing *E. coli*-GFP and stabilised with 5% Tween80 with no (A) or with (B) 0.085 M NaCl in W_2 . Scale bar: 100 μ m.

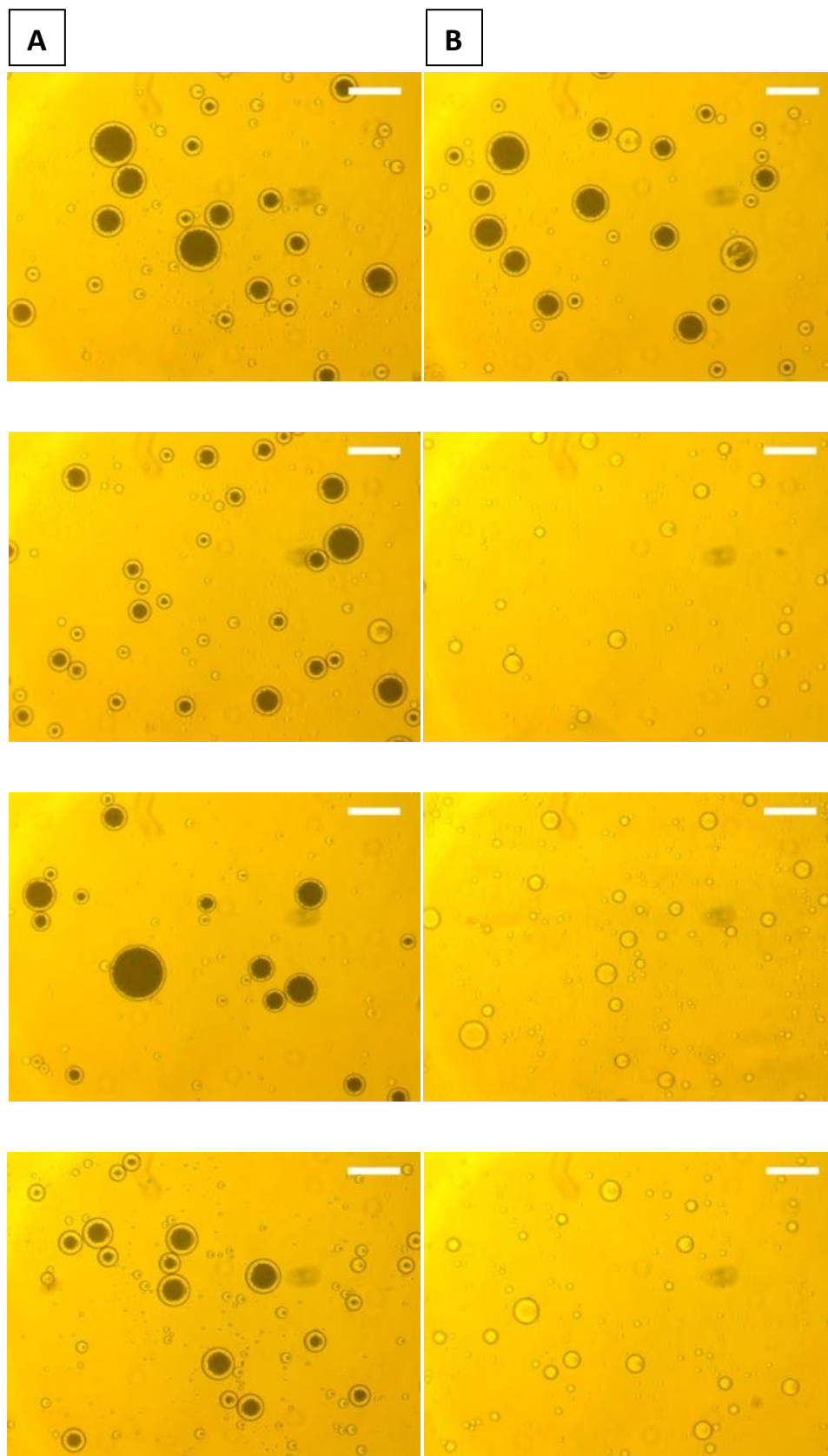


Figure 5.7 Optical microscopy images of $W_1/O/W_2$ emulsions at 0, 2, 4 and 6 hours (from the top). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 40% containing *E. coli*-GFP and stabilised with 1% Tween80 with no (A) or with (B) 0.085 M NaCl in W_2 . Scale bar: 100 μ m.

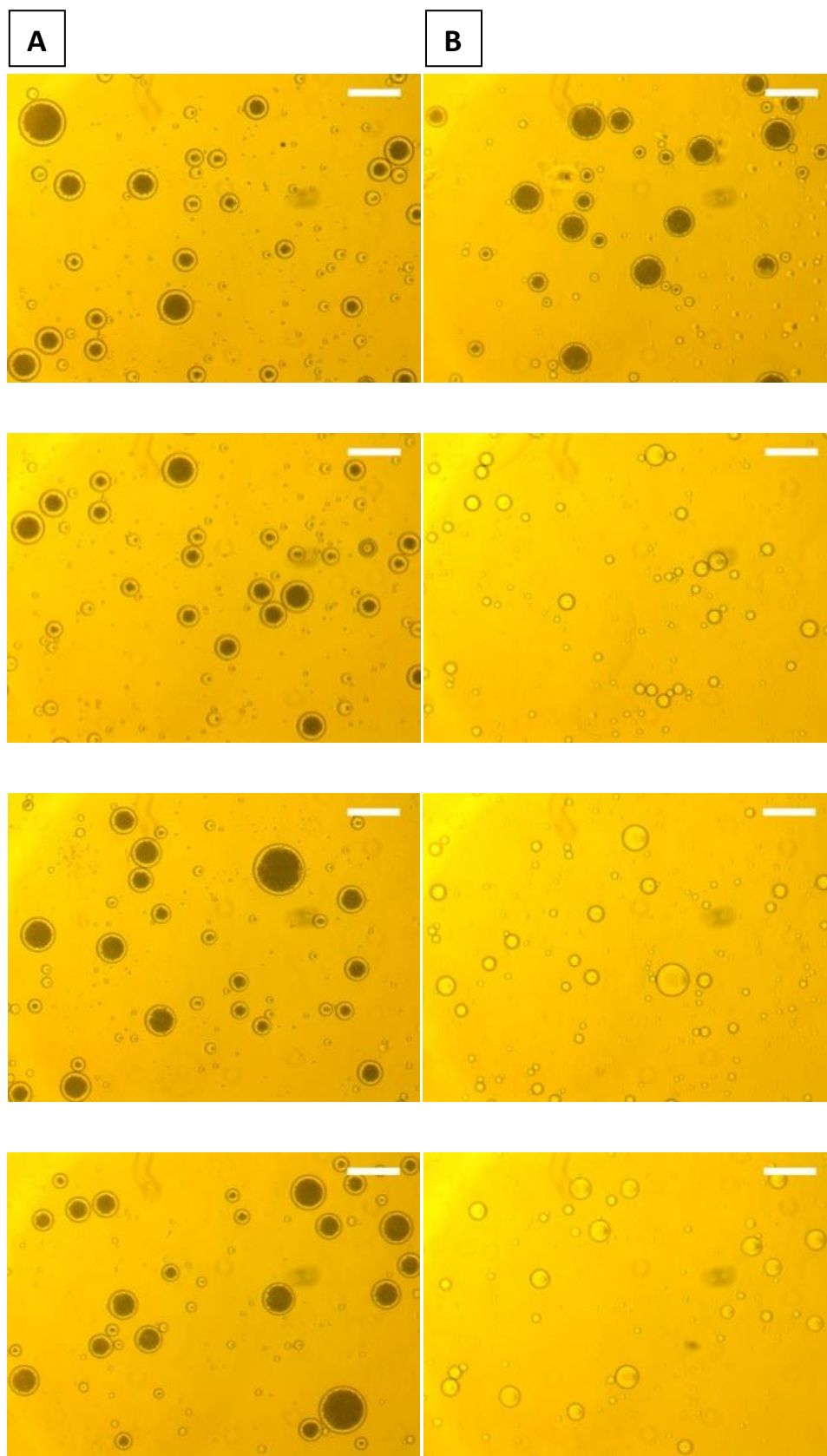


Figure 5.8 Optical microscopy images of $W_1/O/W_2$ emulsions at 0, 2, 4 and 6 hours (from the top). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 40% containing *E. coli*-GFP and stabilised with 5% Tween80 with no (A) or with (B) 0.085 M NaCl in W_2 . Scale bar: 100 μ m.

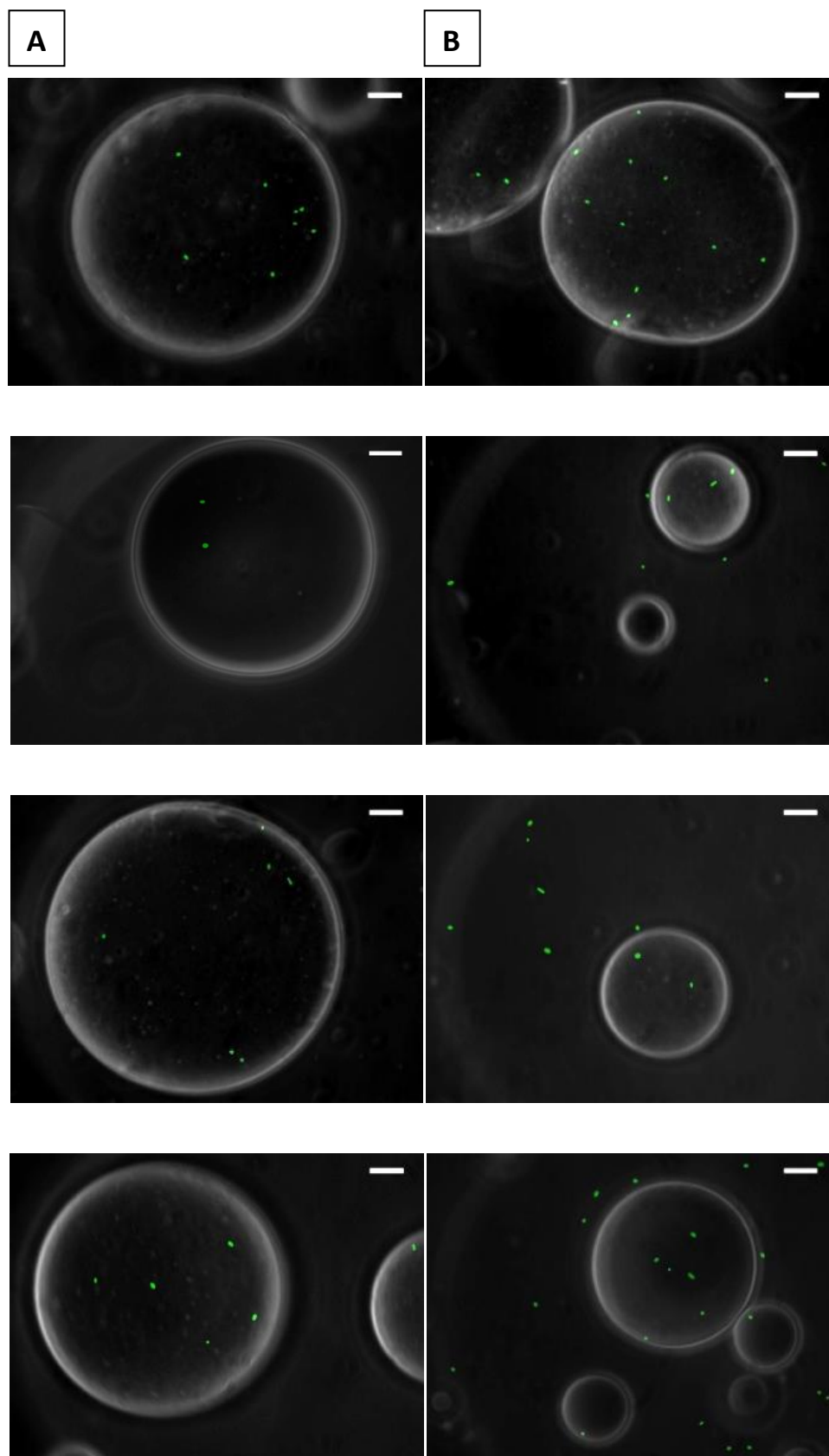


Figure 5.9 Photomicrographs composed from the optical and fluorescence images of *E. coli*-GFP within $W_1/O/W_2$ double emulsions at 0, 2, 4 and 6 hours (from the top). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 20% containing bacteria and stabilised with 1% Tween80 with no (A) or with (B) 0.085 M NaCl in W_2 . Scale bar: 10 μ m.

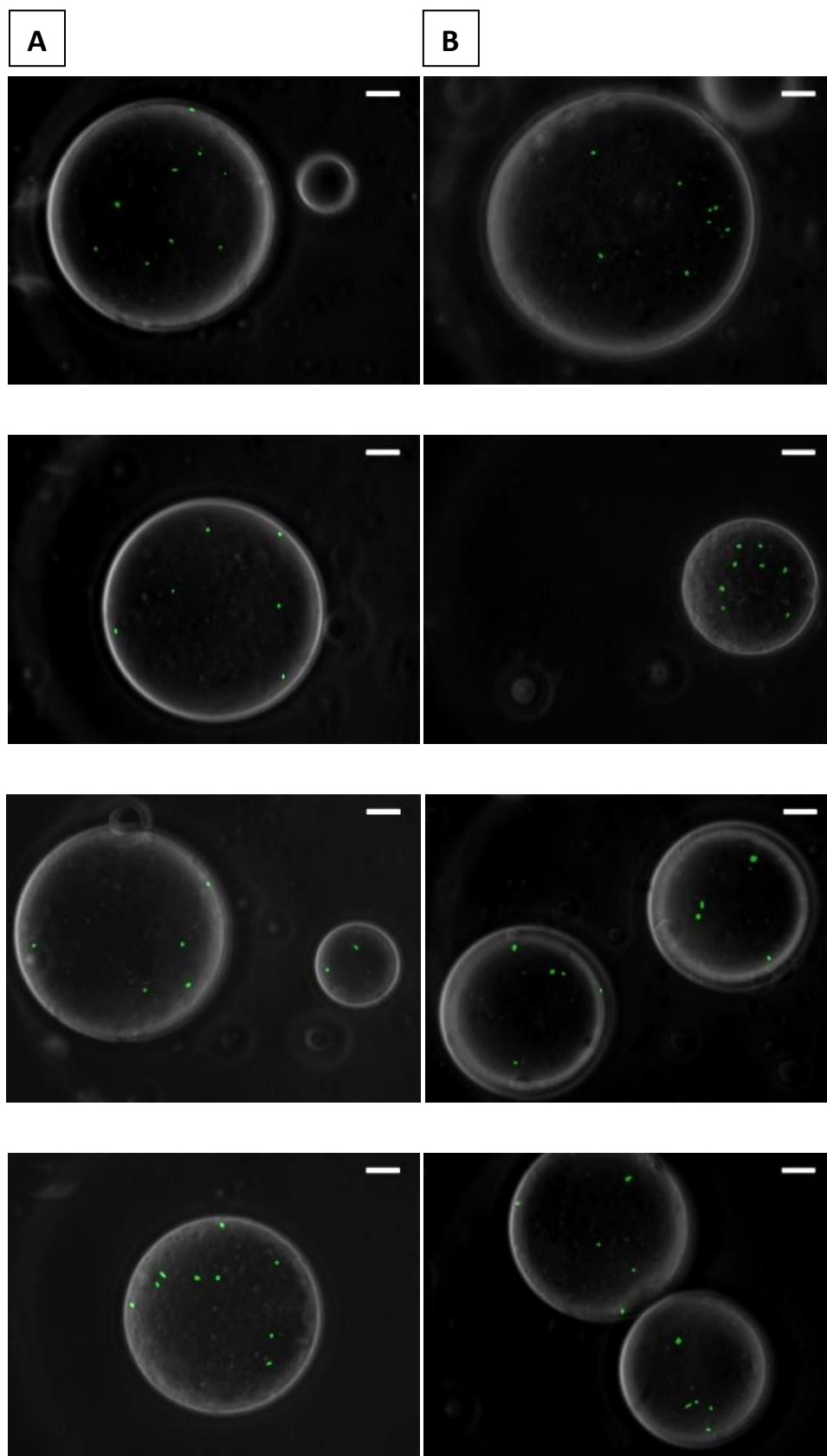


Figure 5.10 Photomicrographs composed from the optical and fluorescence images of *E. coli*-GFP within $W_1/O/W_2$ double emulsions at 0, 2, 4 and 6 hours (from the top). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 20% containing bacteria and stabilised with 5% Tween80 with no (A) or with (B) 0.085 M NaCl in W_2 . Scale bar: 10 μ m.

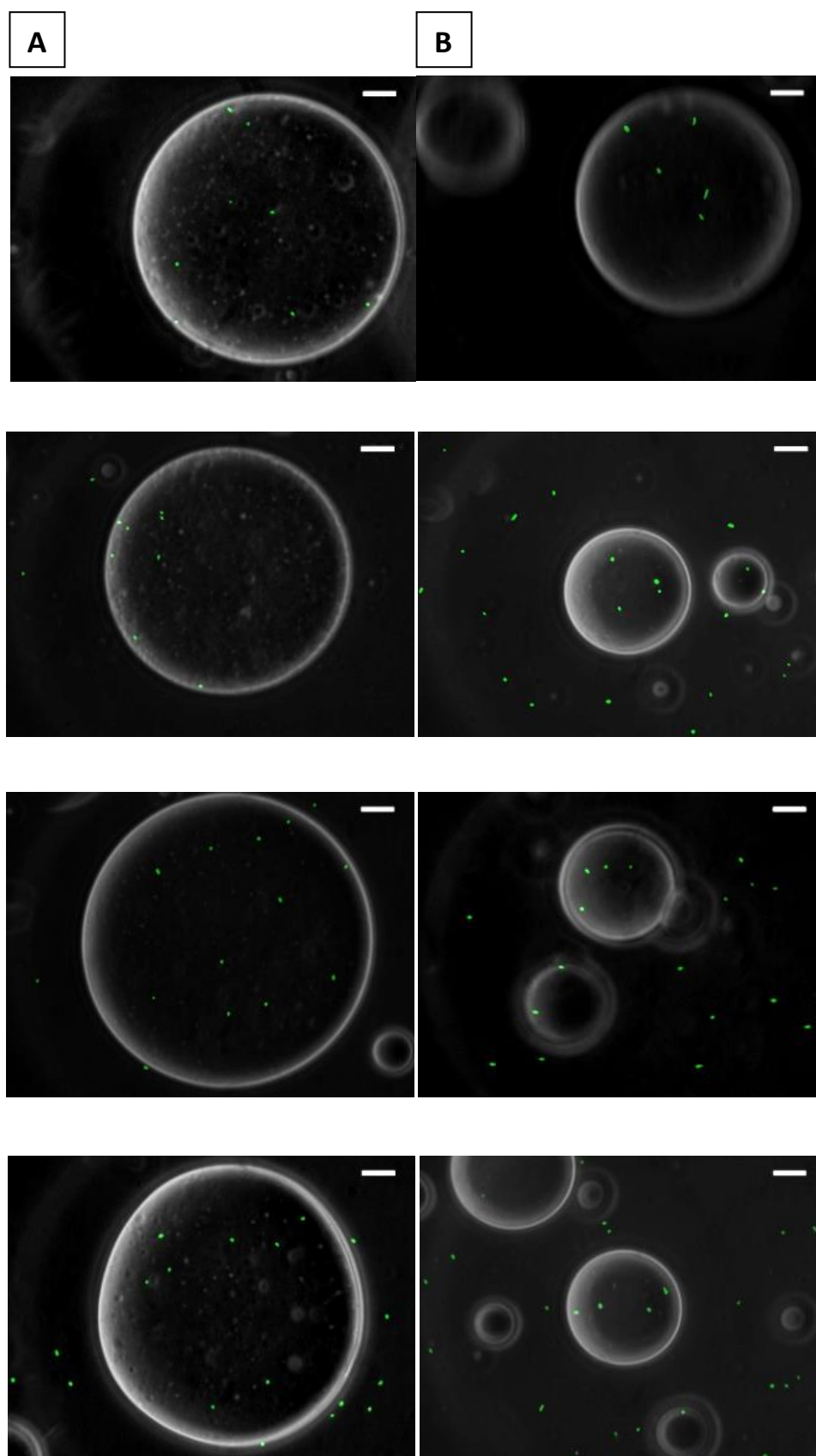


Figure 5.11 Photomicrographs composed from the optical and fluorescence images of *E. coli*-GFP within $W_1/O/W_2$ double emulsions at 0, 2, 4 and 6 hours (from the top). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 40% containing bacteria and stabilised with 1% Tween80 with no (A) or with (B) 0.085 M NaCl in W_2 . Scale bar: 10 μ m.

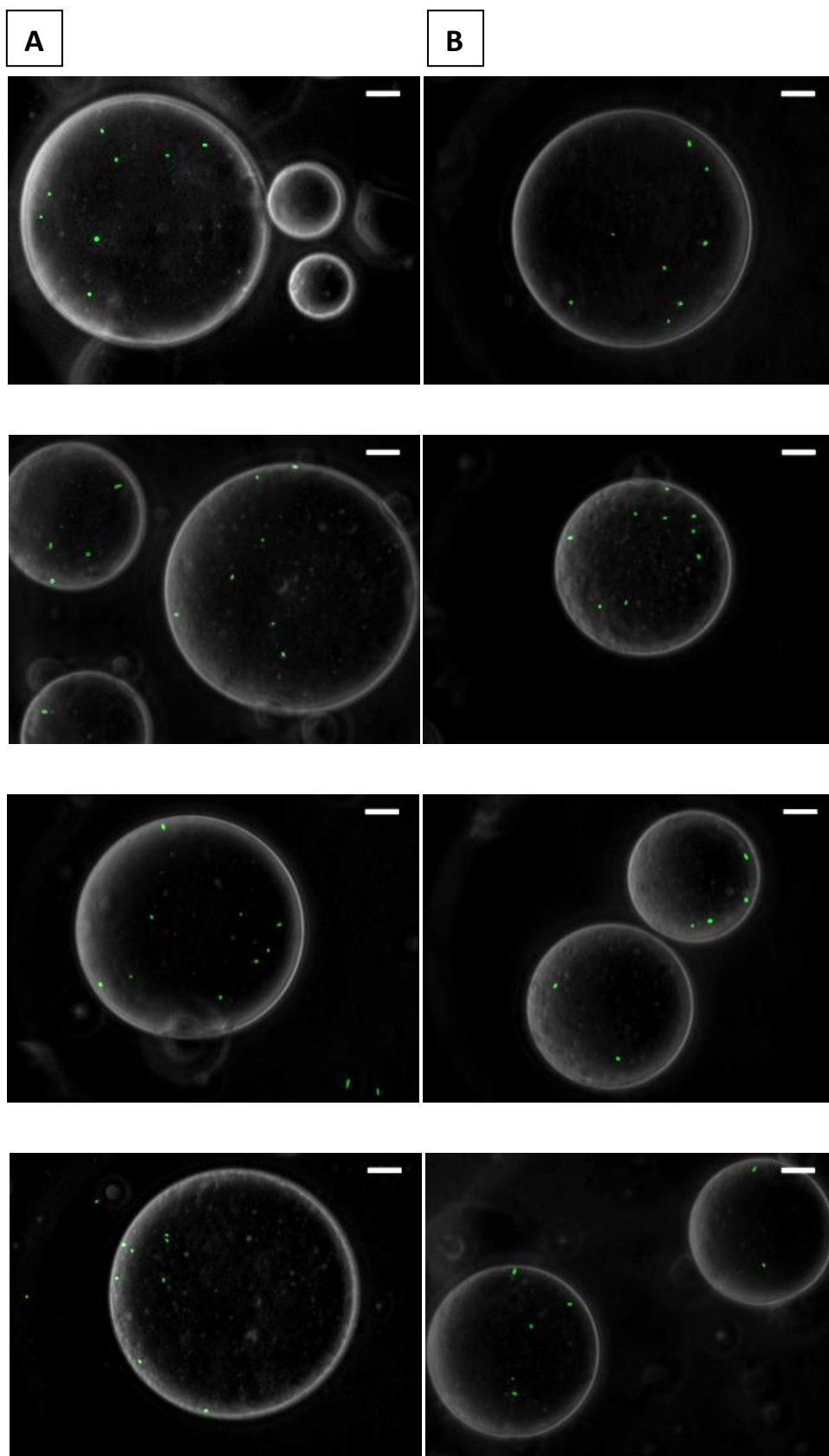


Figure 5.12 Photomicrographs composed from the optical and fluorescence images of *E. coli-GFP* within $W_1/O/W_2$ double emulsions at 0, 2, 4 and 6 hours (from the top). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 40% containing bacteria and stabilised with 5% Tween80 with no (A) or with (B) 0.085 M NaCl in W_2 . Scale bar: 10 μ m.

5.3.6 Changes in oil globule size

To understand how the presence of NaCl in W_2 affected the stability of the oil globules within different formulations of $W_1/O/W_2$ emulsions with or without *E. coli*-GFP, the change in oil globule size [D (4, 3)] over time was measured (Figures 5.13 and 5.14 and Table 5.2).

The presence of bacteria in W_1 had no effect on the D (4, 3) during the incubation period. There was a significant difference ($P<0.05$) in the D (4, 3) with NaCl compared to without NaCl in W_2 . After adding NaCl to W_2 the D (4, 3) significantly ($p<0.05$) decreased (Figures 5.13 and 5.14 and Table 5.2). When the concentration of NaCl in W_2 was low (0.02 M and 0.04 M) the reduction in D (4, 3) was significantly ($P<0.05$) smaller compared to when the concentration of NaCl in W_2 was high (0.085 M and 0.17 M) (Figure 5.13).

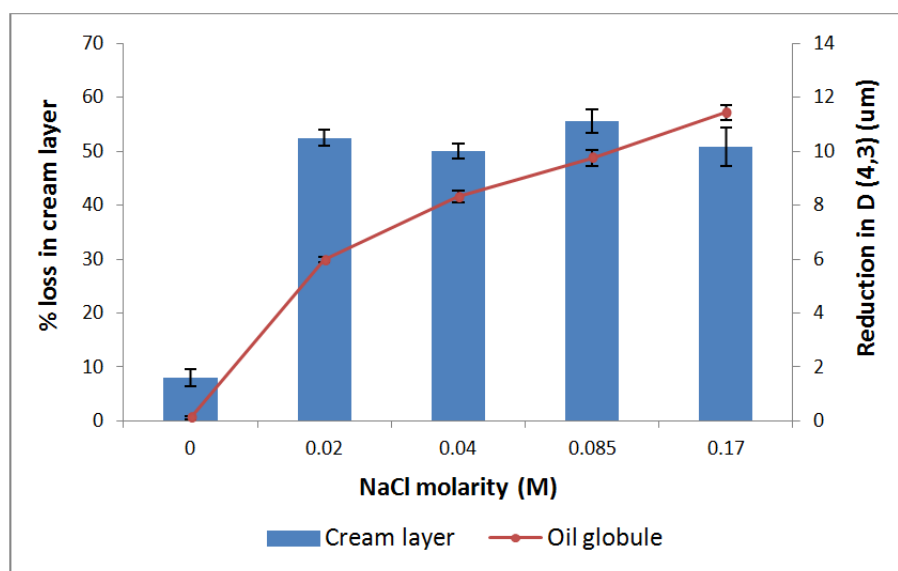


Figure 5.13 Reduction in mean oil globule diameter size (μm) by light scattering [D (4, 3)] and percentage loss in cream layer thickness of $W_1/O/W_2$ emulsions after 1-hour relative to hour 0 incubated at 25°C. The $W_1/O/W_2$ emulsions were prepared with 40% W_1 and stabilised with 1% Tween80 in the presence of bacteria without or with varying concentrations of NaCl (0.02 M, 0.04 M, 0.085 M or 0.17 M) in the W_2 phase. Results are taken from a minimum of 3 independent experiments. The data was analysed with one-way ANOVA.

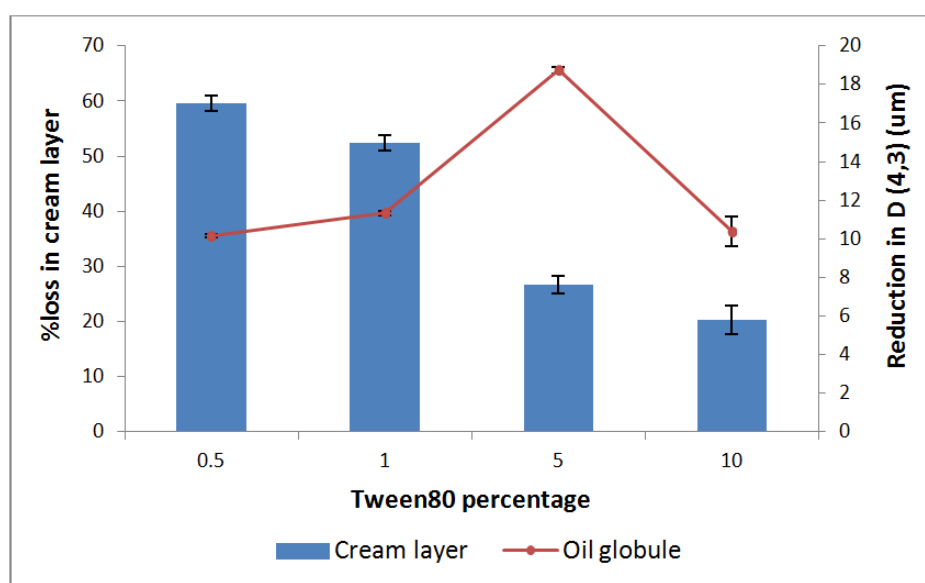


Figure 5.14 Reduction in mean oil globule diameter size (μm) by light scattering [D (4, 3)] and percentage loss in cream layer thickness of $W_1/O/W_2$ emulsions after 1-hour relative to hour 0 incubated at 25°C. The $W_1/O/W_2$ emulsions were prepared with 40% W_1 and stabilised with varying concentrations of Tween80 (0.5%, 1%, 5% or 10%) in the presence of bacteria with 0.085 M NaCl in the W_2 phase. Results are taken from a minimum of 3 independent experiments. The data was analysed with one-way ANOVA.

Table 5.2 Change in mean oil globule diameter size (μm) of $W_1/O/W_2$ emulsions by light scattering [D (4, 3)] after 2, 4 and 6 hours relative to hour 0 incubated at 25°C. The $W_1/O/W_2$ emulsions were prepared with varying concentrations of Tween80 and W_1 in the presence or absence of *E. coli*-GFP with or without 0.085 M NaCl in the W_2 phase. Results are taken from a minimum of 3 independent experiments.

	DE formulations	2 hours	4 hours	6 hours
No <i>E. coli</i> -GFP	20% W_1 , 1% Tw80, no NaCl	1.00 \pm 0.98 ^a	2.58 \pm 2.70 ^a	2.23 \pm 1.70 ^a
	20% W_1 , 1% Tw80, 0.085 M NaCl	14.30 \pm 4.65 ^b	16.70 \pm 4.26 ^b	15.26 \pm 1.80 ^b
	20% W_1 , 5% Tw80, no NaCl	0.16 \pm 1.23 ^a	1.00 \pm 1.25 ^a	-0.03 \pm 1.30 ^a
	20% W_1 , 5% Tw80, 0.085 M NaCl	28.50 \pm 9.10 ^c	28.50 \pm 9.30 ^c	28.20 \pm 10.30 ^c
	40% W_1 , 1% Tw80, no NaCl	0.50 \pm 0.46 ^a	0.70 \pm 0.59 ^a	0.78 \pm 0.26 ^a
	40% W_1 , 1% Tw80, 0.085 M NaCl	22.70 \pm 1.35 ^c	22.90 \pm 0.80 ^c	25.30 \pm 0.80 ^c
	40% W_1 , 5% Tw80, no NaCl	1.20 \pm 0.60 ^a	1.27 \pm 0.60 ^a	1.99 \pm 1.14 ^a
	40% W_1 , 5% Tw80, 0.085 M NaCl	43.60 \pm 2.50 ^d	44.20 \pm 2.93 ^d	43.60 \pm 2.54 ^d
<i>E. coli</i> -GFP	20% W_1 , 1% Tw80, no NaCl	2.40 \pm 1.26 ^a	3.60 \pm 0.64 ^a	2.55 \pm 2.20 ^a
	20% W_1 , 1% Tw80, 0.085 M NaCl	14.50 \pm 2.65 ^b	18.90 \pm 3.10 ^b	18.30 \pm 5.40 ^b
	20% W_1 , 5% Tw80, no NaCl	1.19 \pm 0.33 ^a	1.00 \pm 0.39 ^a	-1.77 \pm 3.30 ^a
	20% W_1 , 5% Tw80, 0.085 M NaCl	30.70 \pm 5.1 ^c	30.80 \pm 4.30 ^c	30.20 \pm 3.80 ^c
	40% W_1 , 1% Tw80, no NaCl	1.37 \pm 0.73 ^a	1.77 \pm 0.96 ^a	3.60 \pm 1.55 ^a
	40% W_1 , 1% Tw80, 0.085 M NaCl	21.00 \pm 1.00 ^c	22.40 \pm 0.64 ^c	24.80 \pm 1.40 ^c
	40% W_1 , 5% Tw80, no NaCl	0.13 \pm 0.17 ^a	1.00 \pm 0.20 ^a	1.10 \pm 0.37 ^a
	40% W_1 , 5% Tw80, 0.085 M NaCl	39.10 \pm 1.96 ^d	41.60 \pm 2.35 ^d	41.00 \pm 2.10 ^d

The data was analysed with one-way ANOVA.

a, b, c, d means \pm standard deviation with different letters are significantly different

5.3.7 Changes in creaming behaviour

To understand how the presence of NaCl in W_2 affects the creaming of $W_1/O/W_2$ emulsions with or without bacteria the change in percentage of loss of cream layer was measured over time (Figures 5.13, 5.14, 5.15A and 5.15B).

The loss of cream layer thickness was significantly ($P<0.05$) increased at 40% W_1 and 1% Tween80 with NaCl compared to without NaCl in W_2 (Figures 5.13, 5.15A and 5.15B) but was similar over the varying concentrations of NaCl (Figure 5.13). After adding NaCl to W_2 the loss of creaming thickness was significantly ($P<0.05$) higher when the concentration of Tween80 was low (0.5% and 1%) compared to when high (5% and 10%) (Figures 5.14, 5.15A and 5.15B).

The presence of bacteria in W_1 had no effect on the cream layer thickness during the incubation period. When no NaCl was added in W_2 and at 20% or 40% W_1 there was no significant difference in loss of cream layer at 1% Tween80 compared to 5% Tween80 (Figures 5.15A and 5.15B). At 1% Tween80 the loss of cream layer was significantly ($P<0.05$) higher with NaCl compared to without NaCl in W_2 regardless of W_1 concentration. Moreover, the loss of cream layer was significantly ($P<0.05$) higher at 1% Tween80 and 40% W_1 compared to 20% W_1 . Interestingly there was no significant difference in loss of cream layer at 20% W_1 and 5% Tween80 with NaCl compared to without NaCl in W_2 .

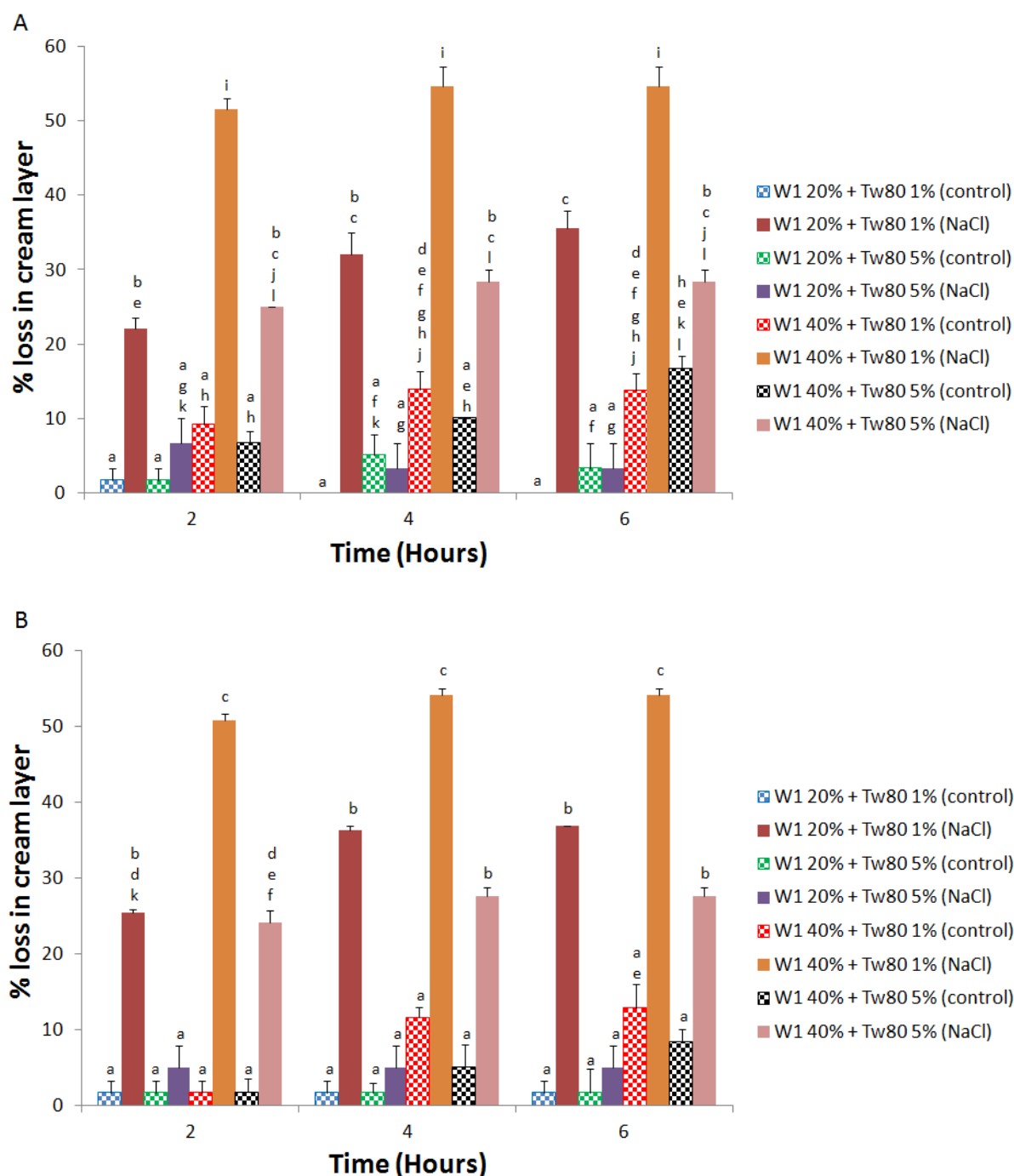


Figure 5.15 Percentage of cream layer loss of $W_1/O/W_2$ emulsions after 2, 4 and 6 hours incubated at 25°C. The $W_1/O/W_2$ emulsions were prepared with different inner-phase (W_1) volume percentage of 20% or 40% with (A) or without (B) *E. coli*-GFP in the W_1 phase and stabilised with 1% or 5% Tween80 with or without 0.085 M NaCl in the W_2 phase. Bars represent mean \pm SEM taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different ($P < 0.05$). The data was analysed with one-way ANOVA.

5.3.8 Effect of encapsulation and release on *E. coli* viability

The effects of $W_1/O/W_2$ emulsion structure and the release mechanism on *E. coli* viability was assessed by plating and microscopic observation staining with PI (Figures 5.16A and B). There was no significant difference in the number of *E. coli* cells obtained from the plating method compared to microscopic enumeration (Figure 5.16A). However, after release into the W_2 phase, 84% of *E. coli* cells were PI positive (Figure 5.16B) even though the bacterial cells were viable suggesting that PI positive cells were not dead. This suggests that the encapsulation process and release mechanism has no effect on *E. coli*-GFP cells.

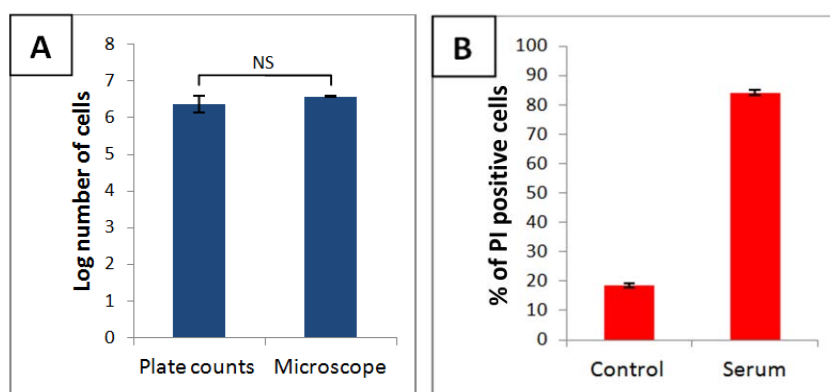


Figure 5.16 Log number of released *E. coli* cells in serum phase quantified by plate counting and microscopic enumeration (A) and percentage of PI positive *E. coli* cells in serum phase and control (B). The $W_1/O/W_2$ emulsion was made with 40% W_1 and stabilised with 1% Tween80 with 0.085 M NaCl in W_2 . Bars represent mean \pm SEM taken from a minimum of 3 independent experiments. The data was analysed with Student's *t*-Test.

5.4 Discussion

As expected, the presence of NaCl in the W_2 phase caused the oil globules to become significantly ($P < 0.05$) smaller in size as NaCl creates an osmotic pressure gradient between the two aqueous phases forcing water to be transported from W_1 to W_2 resulting in oil globule shrinkage (Florence and Whitehill, 1985; Wen and

Papadopoulos, 2001). As water migrates from W_1 to W_2 the change in oil globule size diminishes because of the reduction in the concentration gradient. Also, the reduction in D (4, 3) significantly ($P < 0.05$) increased with higher concentration of NaCl (0.085 M and 0.17 M) compared to a lower concentration of NaCl (0.02 M and 0.04 M). The presence of 0.085 M and 0.17 M NaCl in W_2 creates an osmotic pressure of 42 atm and 84.2 atm respectively whereas with 0.02 M and 0.04 M NaCl in W_2 creates an osmotic pressure of only 10.52 atm and 21 atm, respectively. Also, the reduction in oil globule size was significantly smaller with 5% Tween80 compared to with 1% and 0.5% Tween80 regardless of W_1 concentration indicating that the loss in W_1 was greater with 5% Tween80. Excess Tween80 in the W_2 phase increases the rate of water transport from W_1 to W_2 through mixed reverse micelles and/or surfactant hydration mechanism (Ficheux *et al.*, 1998). Furthermore, the lifetime of W_1 droplets within the oil globules could last from several months to few minutes depending on the concentration of Tween80 (Pays *et al.*, 2002).

Osmotic pressure difference that leads to the collapse of the oil globules was first observed when $W_1/O/W_2$ emulsion entrapping a drug was delivered *in vivo* (Collings, 1971). This immediate collapse of $W_1/O/W_2$ globules was believed to occur due to the higher osmotic pressure of body fluids compared to the inner phase causing shrinking and/or bursting of the oil globules. Since salt in W_2 creates an osmotic pressure gradient which draws water from W_1 to W_2 and bacteria are hydrophilic it was hypothesised that bacterial cells will move from the oil phase to W_2 . Therefore, further investigation was carried out to understand whether it is the bursting or shrinkage of the oil globules being responsible for the release of bacteria and subsequently try to control this mechanism by changing the structure of the $W_1/O/W_2$ emulsion.

In the video-microscopy results it was observed that after adding NaCl in the W_2 phase of the formulation containing low concentration of Tween80 and high amount of the W_1 phase the oil globule bursts releasing W_1 droplets and *E. coli*-GFP into W_2 . NaCl can interact with Tween80 and weaken the interfacial membrane of the oil globule (Jiao *et al.*, 2002; Opawale and Burgess, 1998). Opawale and Burgess (1998) demonstrated that the effect of NaCl on the interfacial elasticity (relates to interfacial film strength) was surfactant specific. The authors showed that for the multiple emulsion formulations that they studied, the interfacial elasticity was reduced with increasing concentrations of NaCl. In another study by Jiao *et al.* (2002) NaCl not only resulted in water loss due to reverse osmosis as did sodium salicylate when added to W_2 but caused a significant reduction in the interfacial elasticity of the film probably due to unfavourable interactions between NaCl and Tween80 and/or salting out (Jiao *et al.*, 2002). Interestingly, after adding NaCl in the W_2 phase of the formulation containing high concentration of Tween80 and low amount of W_1 phase *E. coli*-GFP cells remain within the oil globule despite the loss in W_1 droplets. The solubilisation of *E. coli*-GFP cells within the oil phase was probably due to the presence of PGPR that adsorbs at the bacterial lipid membrane due to its lipophilic nature. It was also observed that the W_1 droplets did not immediately disappear upon release. The W_1 droplets were delivered into the external phase after the oil globule bursts and these droplets persisted in W_2 confirmed by a small peak observed during particle size distribution measurements (Fig A9A, B and C) and microscopic observation of the serum phase (Fig A10). A similar observation was reported by Jiao *et al.* (2002) showing that upon applying force using a coverslip the more stable $W_1/O/W_2$ emulsion globules form structures that had a “dimpled” appearance because of W_1 droplets being pushed to the edge of the oil globule and these were in contact with the continuous phase being

separated by a very thin bio-molecular film with no apparent Becke line (Jiao *et al.*, 2002). However, more investigation is needed to understand the nature of these W_1 droplets that persist within the W_2 phase.

So far, the results showed that the release of *E. coli*-GFP is due to oil globule bursting independent to diffusion mechanism. In this study, it was found that at low concentration of Tween80 the release of *E. coli*-GFP was significantly ($P < 0.05$) increased. The stabilizing effects of hydrophilic surfactants is increased when their concentration is higher in W_2 as they form condensed interfacial films (Tadros, 2013) and strengthen the interfacial film (Jager-Lezer *et al.*, 1997). An increase in the amount of Tween80 adsorbed at the O/W_2 interface produces more condensed interfacial films, able to resist hole creation and propagation that leads to film rupture (Nikiforidis and Kiosseoglou, 2011). It has been reported that interfacial processes control the transport of water in $W_1/O/W_2$ emulsions rather than bulk diffusion (Jager-Lezer *et al.*, 1997). When the W_1 droplets and oil globule interfaces are not physically in contact the spontaneous emulsification and reverse micellisation will be controlling the transport of water between the W_1 and W_2 phases with transport rates independent of NaCl concentration in W_2 (Wen and Papadopoulos, 2001). However, in the presence of NaCl in W_2 and when the W_1 droplet and oil globule interfaces are physically in contact the surfactant molecule hydrates at one interface, diffuse through the oil phase to dehydrate at the other interface that is in contact with the phase of higher solute concentration (Wen and Papadopoulos, 2000a). Moreover, during that process the amount of surfactant molecules at the globule's interface is reduced and this could lead to the interfacial film becoming more susceptible to rupture. At 1% Tween80 the amount of surfactant molecules in the W_2 is not sufficient to adequately replace the migrating surfactant molecules desorbing from

the interface. In contrast at 5% Tween80 the amount of Tween80 in W_2 is sufficient to replace the migrating surfactant molecules from the interface and therefore prevents or slows down the rupture of the interfacial film. Also since more W_1 droplets exist within the oil globule at 40% W_1 compared to 20% W_1 the rate of surfactant hydration mechanism is increased and therefore more droplets would burst leading to the release of *E. coli*-GFP into W_2 . Therefore, it was believed that NaCl caused release of the *E. coli*-GFP cells through destructive interactions on the interfacial film of the oil globules. However, it cannot be dismissed that other mechanisms in addition to globule bursting may have been responsible for the release of *E. coli*-GFP. For example, the presence of NaCl in W_2 screens the electrostatic repulsion between the oil globules (Ficheux *et al.*, 1998), which leads to the increase in coalescence events between the oil globules. Moreover, during this process the release of *E. coli*-GFP to W_2 phase is possible. However, some coalescence events between the oil globules were observed when NaCl was present in W_2 but this process did not lead to the release of *E. coli*-GFP as they remained within the oil globule after coalescence. Another possibility for the release of *E. coli*-GFP would be the increase in coalescence of W_1 droplets with the oil globule interface. However, this release mechanism was shown to increase with increasing amounts of Tween80 (Ficheux *et al.*, 1998) which if was occurring in our study would have resulted in increased release of *E. coli*-GFP at high concentration of Tween80 but that would contrast with what was observed in this study. Further investigation is required to confirm if such mechanism was associated with the release of *E. coli*-GFP from the oil globules along with the bursting mechanism that was observed after adding NaCl in the W_2 phase.

When $W_1/O/W_2$ emulsions are created some of the lipophilic surfactant molecules move to the external O/W_2 interface and influence the stability of multiple emulsion oil globules (Schimdt *et al.*, 2009). The movement from W_1/O interface and adsorption of PGPR at the O/W_2 interface has been documented in $W_1/O/W_2$ emulsions (Schuch *et al.*, 2014). The lipophilic and hydrophilic surfactants at the O/W_2 interface can interfere with each other's stabilizing performance affecting the interfacial film strength. Jiao *et al.* (2002) demonstrated that $W_1/O/W_2$ emulsions formed with Tween80 and Span80 or Span83 had high interfacial film strength but when formed with Tween80 and Span85 or Span60 the interfacial film strength was low. The authors concluded that due to Span85's bulky alkyl chain tail which prevents interactions with Tween80 at the O/W_2 interface led to a decrease in interfacial film strength and that due Span60's higher HLB value (4.7) an unstable $W_1/O/W_2$ emulsion was formed. However, the interaction of PGPR with Tween surfactants at the O/W_2 interface and its effects on interfacial film strength is not yet well understood. Moreover, PGPR can compete with the hydrophilic surfactant at the O/W_2 interface leading to oil globule structural instability (Leal-Calderon *et al.*, 2012). If PGPR may interact with Tween80 it was believed that at low concentration of Tween80 (1% wt) in W_2 more PGPR molecules could absorb at the O/W_2 interface which may have resulted in reduced interfacial film strength and/or unfavourable interactions with Tween80 eventually weakening the interfacial film making it more susceptible to rupture. However, further studies are required to test if this hypothesis is true.

At high concentration of W_1 the release of *E. coli*-GFP increased. Even though the concentration of PGPR was kept at 2% in this study the amount of PGPR required for full surface coverage of the W_1 droplets interface is higher in $W_1/O/W_2$ emulsion

containing 40% W_1 compared to 20% W_1 . This will result in less amount of excess PGPR in the oil phase at 40% W_1 than at 20% W_1 . Our results show that increasing the PGPR concentration increases the viscosity of the emulsion (Table 5.1). Jiao *et al.* (2002) demonstrated that an increase in oil phase viscosity due to increase in the concentration of Span80 results in less deformation of double emulsion oil globules upon applying force (coverslip). Furthermore, the presence of excess lipophilic surfactant in the oil phase was found to increase the visco-elasticity of the interfacial film of the oil globule (Vasiljević *et al.*, 2006). From our results, the viscosity of W_1/O was significantly higher with 40% W_1 compared to 20% W_1 . According to the Mooney equation (Mooney, 1951; Kita *et al.*, 1977) higher W_1 fraction increases the viscosity of the oil globule and this in turn leads to its destabilisation and increases the likelihood of rupture of the W_1 droplets and oil globules (Jiao and Burgess, 2003). However, this increase in viscosity has an opposite effect on emulsion stability than does the viscosity increase due to increased lipophilic surfactant in the oil phase (Jiao and Burgess, 2003).

So far, these results showed that the release of *E. coli*-GFP follows a similar pattern as changes in loss of cream layer independent to changes in D (4, 3). It was believed that the cream layer thickness was related to the amount of oil globules in the emulsion and the increase in loss of cream layer thickness indicates loss of oil globules. The presence of *E. coli*-GFP within the $W_1/O/W_2$ emulsion globules had no effect on the transport of water and/or the bursting of the oil globules. Since bacteria are living organisms and they secrete waste products a change in osmotic pressure inside the oil globules is expected (Boitard *et al.*, 2012). However, with the bacterial cell concentration and the conditions that were used in this study *E. coli*-GFP showed no significant difference compared to without *E. coli*-GFP on the change in D

(4, 3) or creaming stability presumably due to relatively low metabolism and thus low waste production. Also there was no effect on viability of *E. coli* cells after being released from the $W_1/O/W_2$ emulsion. However, there was a significant increase in PI positive *E. coli* cells after release compared to control. Non-ionic surfactants can enhance the fluidity of the bacterial membrane and hence increase its permeability (Glover *et al.*, 1999). Since PI is a membrane integrity indicator (Zhang and Crow, 2001) and in this study the number of PI stained cells do not correlate with cell viability data it is most probable that the surfactants increased the fluidity and permeability of the bacterial membrane allowing PI to permeate and bind to nucleic acids. Also, NaCl may affect the bacterial membrane. In a recent study by Gandhi and Shah (2015) it was shown that 5% NaCl had a damaging effect on membrane integrity after 1 hour allowing PI to penetrate bacterial cells. Therefore, upon release into the W_2 phase *E. coli* cells were exposed to NaCl and this could have further compromised membrane integrity allowing PI to permeate and stain the cells.

5.5 Conclusion

In this study, the mechanism by which bacterial cells are released from $W_1/O/W_2$ emulsions has been demonstrated for the first time. Using video-microscopy it was shown that the release of bacterial cells was due to oil globule bursting independent to diffusion. This release mechanism has been demonstrated to be modulated by modifying the structure of the $W_1/O/W_2$ emulsion. The release of *E. coli*-GFP was higher at low concentrations of Tween80 and high volumes of W_1 after adding NaCl in the W_2 phase. Moreover, when the concentration of Tween80 was high and the volume of W_1 was low no release occurred after adding NaCl in the W_2 phase. The release of *E. coli*-GFP was facilitated by the addition of NaCl probably due to interfacial processes. Therefore, when formulating $W_1/O/W_2$ emulsions for release

applications it is important to consider the role of the structure and stability of the oil globule's interface. Although the mechanism by which bacteria are released from $W_1/O/W_2$ emulsions after adding NaCl in the W_2 phase was observed further investigation is required to understand the effect of NaCl on the interfacial film of the oil globule for example measuring the visco-elastic properties of the different formulations investigated. Understanding how release can be controlled allows different $W_1/O/W_2$ emulsion formulations for various industrial applications. For example, a fermentation process can contain a secondary bacterial species within the oil globules that can be released in a controlled manner over time. This can reduce the risk of contamination associated with introducing the secondary bacterial species after starting the fermentation process. Also by changing the structure of $W_1/O/W_2$ emulsions the oil globules encapsulating pH sensitive drugs or probiotics can be made more resistant to bursting when ingested or injected *in vivo*.

CHAPTER 6: UNDERSTANDING AND CONTROLLING THE RELEASE

MECHANISM OF *ESCHERICHIA COLI* IN DOUBLE $W_1/O/W_2$ EMULSION

GLOBULES UNDER HYPO-OSMOTIC PRESSURE

6.1 Introduction

Manipulating the osmotic pressure in $W_1/O/W_2$ emulsions can be used for delivering microbial species *in vitro* or *in vivo* which have great potential for applications within the fermentation, agricultural and medical industries. In Chapter 4 the mechanism by which *E. coli* is released from $W_1/O/W_2$ emulsion globules in the presence of NaCl in W_2 was described and it was found that by changing the emulsion structure this mechanism can be controlled. However, there is a lack of studies investigating the release of bacteria from the W_1 phase of $W_1/O/W_2$ emulsions in hypo-osmotic conditions which as opposed to hyper-osmotically triggered release formulations provides a sustained release of salts as well as bacterial cells over a period of time in a controlled manner which is desirable in certain applications.

It was shown that by diluting $W_1/O/W_2$ emulsions in hypo-osmotic solution they swell and break down to release antiseptics (Tedajo *et al.*, 2005). The concentration of the lipophilic (in the oil phase stabilising the W_1/O interface) and hydrophilic (in the W_2 phase stabilising the O/W_2 interface) surfactants play a major role in controlling the release of the water-soluble substances from the W_1 phase of $W_1/O/W_2$ emulsion globules under hypo-osmotic pressure (Geiger *et al.*, 1998; Geiger *et al.*, 1999). When the W_1 volume fraction was increased the release of solutes from the oil globules in hypo-osmotic solution become higher (Cárdenas and Castro, 2003). Also in a hypo-osmotic solution the release of solutes from $W_1/O/W_2$ globules was higher when the osmotic pressure gradient between the W_1 and W_2 phases was increased

(Geiger *et al.*, 1998; Geiger *et al.*, 1999; Cárdenas and Castro, 2003). However, in these studies the mechanism behind the release has not been clear and was described as “thinning of the oily phase” until breakdown of the oil globules occurred (Lutz *et al.*, 2009b). Furthermore, it is difficult to relate the release of water-soluble substances to one mechanism and many processes can be involved including:

- coalescence of the W_1 droplets with O/W_2 interface (Ficheux *et al.*, 1998),
- rupture of the O/W_2 interfacial film (Geiger *et al.*, 1998),
- migration of the water and water-soluble substances through the oil phase via surfactant-facilitated transport through reverse micelles (Colinart *et al.*, 1984) hydrated surfactant and/or diffusion through thin surfactant lamellae (Omotosho *et al.*, 1986).

In the previous chapter, it was shown that the release of bacteria from $W_1/O/W_2$ emulsion globules under hyper-osmotic pressure resulted from bursting of the oil globules independent of diffusion mechanisms. Moreover, investigating the mechanism of release by swelling using insoluble particles such as bacteria as opposed to water-soluble substances in W_1 is advantageous when trying to understand how release occurs during the swelling process of the $W_1/O/W_2$ globules under hypo-osmotic pressure. This is because the release of water-soluble substances from $W_1/O/W_2$ globules can be the result of various mechanisms whereas bacterial release will result from the rupturing of the interfacial film and bursting of the oil globules. Therefore, the mechanism responsible for the release of bacteria from $W_1/O/W_2$ globules in a hypo-osmotic solution was investigated and subsequently try to control this mechanism by changing the structure of the $W_1/O/W_2$ emulsion.

The presence of *E. coli* did not affect the stability of $W_1/O/W_2$ emulsion in hyper-osmotic conditions. However, the stability of $W_1/O/W_2$ emulsion in hypo-osmotic conditions has not yet been investigated. In this study, the effect of varying the structure of $W_1/O/W_2$ emulsion on the release of *E. coli* from the oil globules facilitated by osmotic pressure was investigated and the mechanism of this release was microscopically visualised. Also, the amount of inner W_1 phase, hydrophilic surfactant (in W_2 phase) and lipophilic surfactant (in oil phase) were changed and their effect on $W_1/O/W_2$ emulsion stability in the presence of *E. coli*-GFP was analysed. Furthermore, the effects of encapsulation, release, prolonged storage and chemistry of emulsion on *E. coli* was investigated. Also, the effect of the presence of *E. coli* (in W_1 phase) on stability of the $W_1/O/W_2$ emulsion was studied and the encapsulation efficiency of the bacteria was monitored during storage.

6.2 Materials and Methods

6.2.1 Materials

Includes all the materials in Chapter 4, Section 4.2.1, with the addition of the following:

- DiBAC₄ (3) was purchased from Sigma-Aldrich (United Kingdom).

6.2.2 Microbial cultures

Bacterial cultures used and procedure as in Chapter 5, Section 5.2.2.

6.2.3 Preparation of $W_1/O/W_2$ emulsions

Double $W_1/O/W_2$ emulsions were prepared using a high shear mixer homogeniser (Silverson L5M) at room temperature using a two-step emulsification (Appendix, Figure 10.1). In the first step, primary W_1/O emulsions were made. An oil phase was

prepared by dissolving PGPR (2, 4, 6 or 8 wt%) in sunflower oil. The inner aqueous phase (W_1) consisting of de-ionised water was emulsified (120 seconds) into the oil phase (W_1 :O phase ratio of 20:80 or 40:60). For microbial-encapsulation de-ionised water or NaCl solution (final concentration of 0.02 M, 0.04 M, 0.085 M or 0.17 M) containing the washed *E. coli* cells (5×10^7 CFU/ml) was used as W_1 . In the second step, a W_1 /O/ W_2 emulsion was made. The outer aqueous phase (W_2) was prepared by dissolving Tween80 (0.5, 1, 5 or 10 wt%) in de-ionised water at 60°C for approximately 15 minutes. The previously prepared primary W_1 /O emulsion was emulsified (60 seconds) into W_2 to form the W_1 /O/ W_2 emulsion (W_1 :O: W_2 ratio of 20:80). The rotational speeds for homogenising the different formulations of primary W_1 /O and W_1 /O/ W_2 emulsions are shown in Appendix, Table A3. From a volume of 50ml W_1 /O/ W_2 emulsion the cream layer containing the oil globules was separated using a 50ml graduated syringe and the serum phase was discarded. The cream layer was transferred into a sterile Erlenmeyer flask (500 ml) containing Tween80 (0.5, 1, 5 or 10 wt%) dissolved in either de-ionised water or NaCl solution (final concentration of 0.02 M, 0.04 M, 0.085 M or 0.17 M) and incubated at 25°C for 45 minutes or over a 180-minute period shaking at 100 rpm to ensure homogenised mixing.

6.2.4 Characterisation of W_1 /O/ W_2 emulsion stability

The stability of the W_1 /O/ W_2 emulsions was monitored by measuring the oil globule size [D (4, 3)] and creaming thickness during incubation.

6.2.4.1 Measurement of oil globule size of double W_1 /O/ W_2 emulsions

The particle size distribution of oil globules in the W_1 /O/ W_2 emulsion was measured immediately after preparation and as a function of storage time using a laser

diffraction particle size analyser (Malvern Mastersizer 2000, Malvern Instrument Ltd, Worcestershire, UK), equipped with a He-Ne laser ($\lambda = 633$ nm). This procedure was done as in Section 3.2.6.1.

6.2.4.2 Observation of phase separation

The cream height fraction of the $W_1/O/W_2$ emulsion was measured immediately after preparation and as a function of storage time. This procedure was done as in Section 3.2.6.2.

6.2.5 Microscopic observation of the $W_1/O/W_2$ emulsions

Video microscopy was used to observe the release mechanism of *E. coli*-GFP cells from $W_1/O/W_2$ emulsion globules. Fluorescent and optical microscopy was used to monitor *E. coli*-GFP cells in $W_1/O/W_2$ emulsions during incubation.

6.2.5.1 Video time-lapse for observation of oil globule bursting and tracking of *E. coli*-GFP

For video microscopy, the sample was placed on a microscope slide and the video was recorded under objective lens 40x magnification with a Moticam 10 camera via Motic Images Plus video acquisition software at 17fps. The light source used to excite the GFP was a light-emitting diode (LED) and the emission was observed at 509 nm.

6.2.5.2 Fluorescent and optical imaging of the $W_1/O/W_2$ emulsions and *E. coli*-GFP

The double $W_1/O/W_2$ emulsions with or without *E. coli*-GFP were observed using optical and fluorescent microscopy (Zeiss Axioplan) at room temperature. For optical

microscopy imaging the sample was placed on a microscope slide and the image was acquired under objective lens 10x or 100x magnification with a digital colour camera system Motic Moticam 10 using a 10 megapixel CMOC camera via Motic Images Plus video acquisition software. For fluorescent microscopy imaging the sample was placed on a microscope slide and the image was acquired under objective lens 40x magnification with a digital colour camera system Motic Moticam 10 using a 10 megapixel CMOC camera via Motic Images Plus video acquisition software. The light source used to excite the GFP was a LED and the emission was observed at 509 nm.

6.2.6 Measuring encapsulation efficiency (%) and quantifying of the release of *E. coli*-GFP during emulsification and storage.

The number of *E. coli* cells in the serum phase was measured immediately after preparation and as a function of storage time. This procedure was done as in Section 5.2.6.

6.2.7 Determining the total cell count and health of *E. coli* (MG1655) after encapsulation and release using epifluorescence microscopy

E. coli MG1655 in serum phase or de-ionised water (control) was stained by adding DAPI (4 µl/ml) and [DiBAC₄ (3)] (50 ng/ml) and incubated in the dark for 30 minutes. The rest of this procedure was done as in Section 5.2.8.

6.2.8 Statistical analysis

Statistical analysis was done as described in Section 3.2.10 and each experiment was conducted in triplicate (N=3).

6.3 Results

6.3.1 Primary W₁/O emulsion characterization

The size of the W₁ droplets can influence the stability of W₁/O/W₂ emulsions (Weiss and Muschiolik, 2007). In this study, the homogenisation conditions were such that the average mean size distribution [D (4, 3)] of W₁ droplets was comparable (3-5µm; Appendix, Figures A11 and A12) in all primary W₁/O formulations.

6.3.2 Encapsulation efficiency (%) of *E. coli*-GFP cells in W₁/O/W₂ emulsions

The bacterial counts for *E. coli*-GFP before and after the encapsulation process were used to calculate the encapsulation efficiency from different W₁/O/W₂ emulsions. The results indicated successful entrapment of viable bacterial cells (>99.8%) in all the prepared W₁/O/W₂ emulsions (Appendix, Table A4).

6.3.3 Influence of osmotic balance alteration on release of *E. coli*-GFP

To understand how the release of bacteria during osmotic pressure alteration is affected by varying the W₁/O/W₂ emulsion composition, the release of *E. coli*-GFP over time was quantified while altering the osmotic pressure gradient (Figure 6.1A), the concentration of Tween80 (Figure 6.1B), PGPR (Figure 6.1C) and the amount of W₁ phase (Figure 6.2).

With the formulation containing 40% W₁ and stabilised with 1% Tween80 the release of *E. coli*-GFP was significantly ($P<0.05$) increased when osmotic balance was altered compared to when unaltered (Figure 6.1A). The release of *E. coli*-GFP was significantly ($P<0.05$) increased at higher (0.17 M and 0.085 M) compared to lower concentrations (0.04 M and 0.02 M) of NaCl (Figure 6.1A). During osmotic balance

alteration, the release of *E. coli*-GFP was significantly ($P<0.05$) higher at low concentrations of Tween80 (0.5% and 1%) compared to high concentrations of Tween80 (5% and 10%) (Figure 6.1B). During osmotic balance alteration, the release of *E. coli*-GFP was significantly ($P<0.05$) higher at low concentrations of PGPR (2% and 4%) compared to high concentrations of PGPR (6% and 8%) (Figure 6.1C).

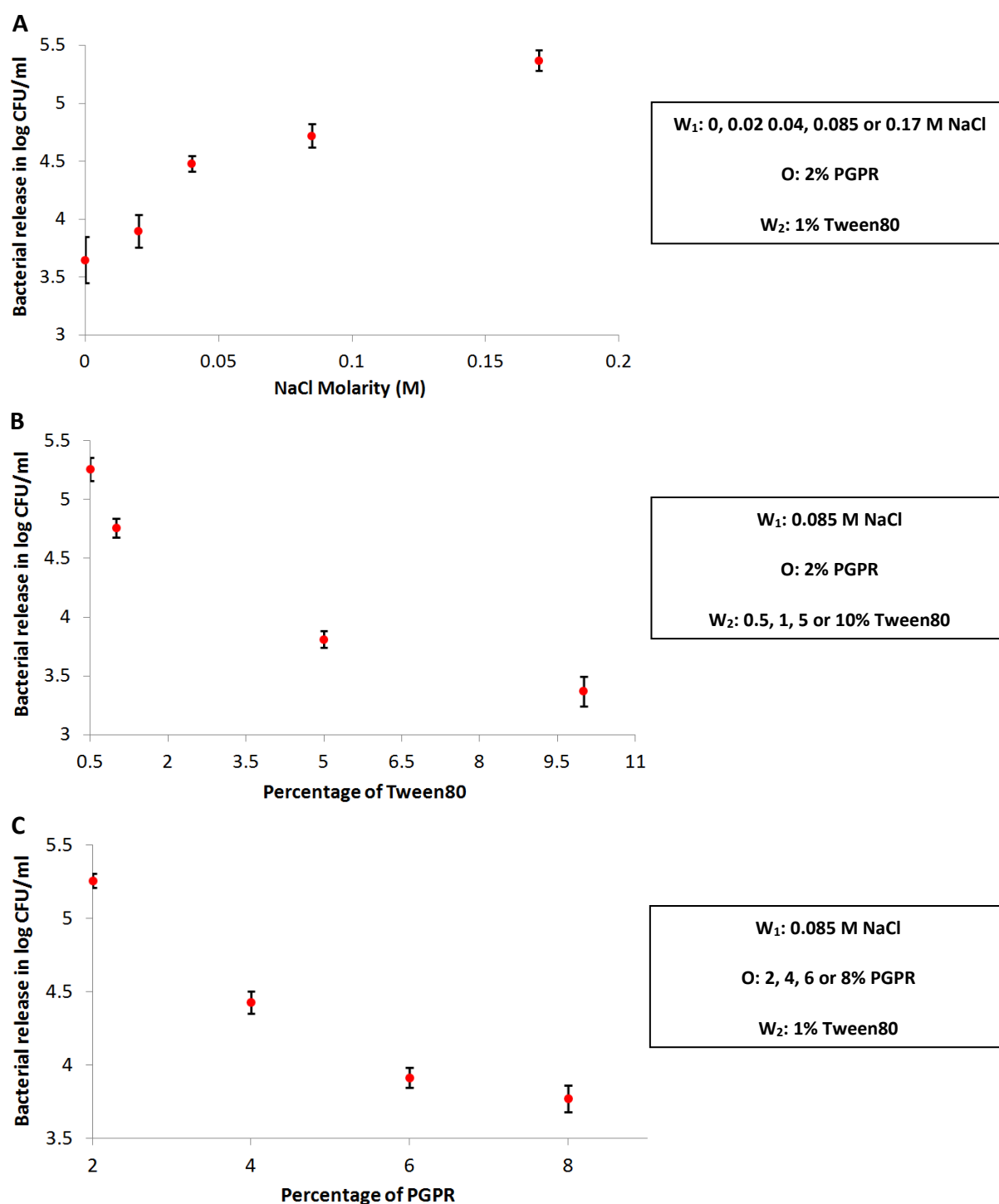


Figure 6.1 Effect of varying the structure of the $W_1/O/W_2$ emulsion on the release of *E. coli*-GFP. The amount of released bacteria in the outer W_2 phase of the $W_1/O/W_2$ emulsions was measured after 45-minutes incubation at 25°C. The $W_1/O/W_2$ emulsions were prepared: A) 40% W_1 containing *E. coli*-GFP and varying concentrations of NaCl (0.02 M, 0.04 M, 0.085 M or 0.17 M) in W_1 and stabilised with 1% Tween80 in W_2 and 2% PGPR; B) 40% W_1 containing *E. coli*-GFP and 0.085 M NaCl in W_1 and and stabilised with varying concentrations of Tween80 (0.5%, 1%, 5% or 10%) in W_2 and 2% PGPR in the oil phase; C) 40% W_1 containing *E. coli*-GFP and 0.085 M NaCl in W_1 and and stabilised with 1% Tween80 in W_2 and varying concentrations of PGPR (2%, 4%, 6% or 8%) in the

oil phase. Bars represent mean \pm SEM taken from a minimum of 3 independent experiments. The data was analysed with one-way ANOVA.

The effect of varying the concentration of W_1 and Tween80 on the release of *E. coli*-GFP with and without altering the osmotic balance over time was quantified (Figure 6.2). The release of *E. coli*-GFP significantly ($P<0.05$) increased during osmotic balance alteration especially when the concentration of Tween80 was low and/or W_1 was high. At 1% Tween80 and regardless of W_1 concentration the release of *E. coli*-GFP was significantly ($P<0.05$) higher when the osmotic balance was altered compared when unaltered (Figure 6.2). After altering the osmotic balance, the release of *E. coli*-GFP was significantly ($P<0.05$) higher at 1% Tween80 when W_1 was 40% compared to 20%. These results suggest that the release of *E. coli*-GFP is affected by the concentration of NaCl, Tween80 and PGPR during osmotic balance alteration.

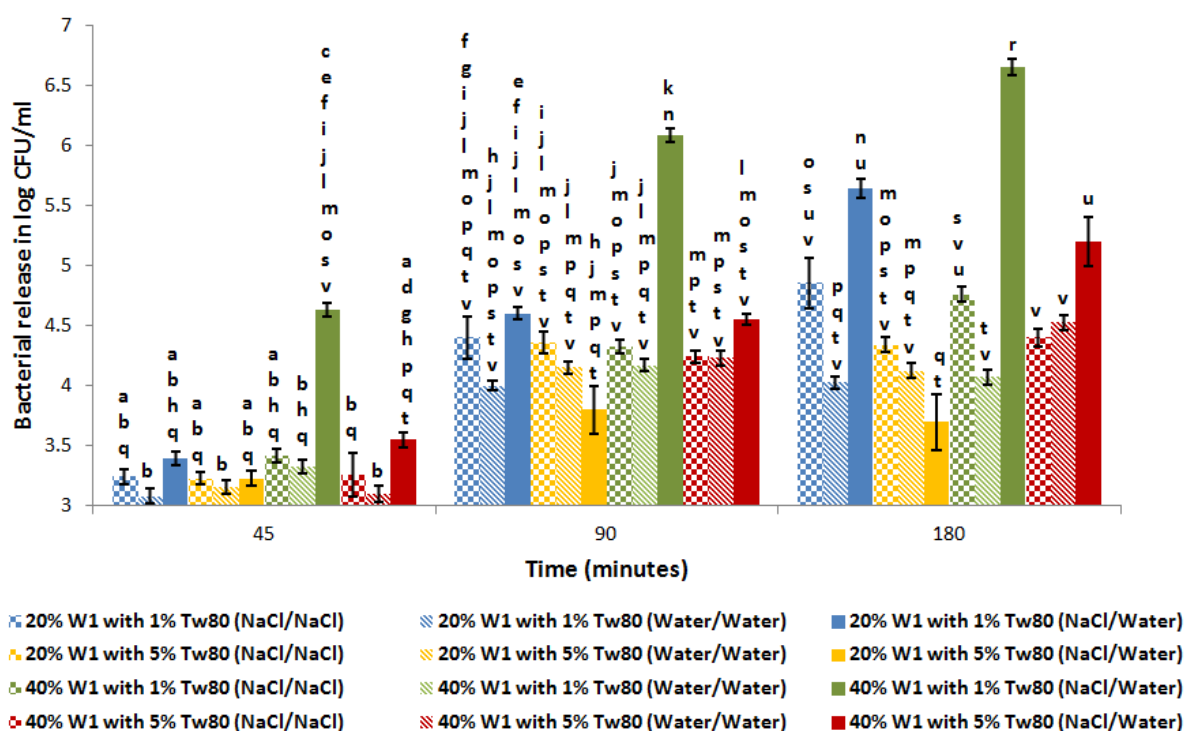


Figure 6.2 Effect of varying the structure of the $W_1/O/W_2$ emulsion on the release of *E. coli*-GFP. The amount of released bacteria in the outer W_2 phase of the $W_1/O/W_2$ emulsions after 45, 90 and 180-minutes incubation at 25°C was quantified. The $W_1/O/W_2$ emulsions were prepared with different inner W_1 phase volume percentage of 20% or 40% containing *E. coli*-GFP with or without 0.085 M NaCl in W_1 and stabilised with 1% or 5% Tween80 in W_2 and 2% PGPR in the oil phase. Bars represent mean \pm SEM taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different ($P < 0.05$). The data was analysed with one-way ANOVA.

6.3.4 Changes in oil globule size during osmotic balance alteration and storage

To understand how the stability of $W_1/O/W_2$ globules encapsulating *E. coli*-GFP is affected during osmotic pressure alteration the change in oil globule size ($D(4, 3)$) was measured over time by varying the osmotic pressure gradient (Figure 6.3A), changing the concentration of Tween80 (Figure 6.3B) and PGPR (Figure 6.3C). The effect of varying the concentration of W_1 and Tween80 on the size ($D(4, 3)$) of the oil

globules with and without altering the osmotic balance in the presence or absence of *E. coli*-GFP in W_1 (Table 6.1) was measured.

The presence of bacteria in W_1 had no effect on the $D(4, 3)$ during the incubation period (Table 6.1). After alteration of the osmotic balance the $D(4, 3)$ significantly ($p < 0.05$) increased (Table 6.1) over time. When the concentration of NaCl in W_1 was high (0.085 M and 0.17 M) the increase in $D(4, 3)$ was significantly ($P < 0.05$) higher compared to when the concentration of NaCl in W_1 was low ((0.02 M and 0.04 M) (Figure 6.3A). During osmotic balance alteration, there was no significant difference in $D(4, 3)$ at different concentrations of Tween80 (Figure 6.3B) or PGPR (Figure 5.3C) but the $D(4, 3)$ was significantly ($p < 0.05$) higher at 40% W_1 compared to 20% W_1 (Table 5.1). There was no significant difference in the $D(4, 3)$ when the osmotic balance was unaltered in between W_1 and W_2 (Table 5.1). These results indicate that the $D(4, 3)$ is affected by the concentration of NaCl and W_1 during osmotic balance alteration whilst the concentration of Tween80 and PGPR had no effect on $D(4, 3)$.

Table 6.1 Change in mean oil globule diameter size (μm) of $W_1/O/W_2$ emulsions by light scattering [$D(4, 3)$] after 45, 90 and 180-minutes relative to time 0 minutes incubated at 25°C. The $W_1/O/W_2$ emulsions were prepared with different inner-phase (W_1) volume percentage of 20% or 40% in the presence or absence of *E. coli*-GFP with or without 0.085 M NaCl in the W_1 phase and stabilised with 1% or 5% Tween80 in W_2 and 2% PGPR in the oil phase. Results are taken from a minimum of 3 independent experiments.

	$W_1/O/W_2$ emulsion formulations	Oil droplet size $D(4, 3)$ (μm)		
		45 minutes	90 minutes	180 minutes
	20% W_1 , 1% Tw80, 0.085 M NaCl in W_1 only	$7.44 \pm 0.40^{\text{ac}}$	$13.66 \pm 0.30^{\text{c}}$	$24.58 \pm 1.20^{\text{d}}$
	20% W_1 , 1% Tw80, 0.085 M NaCl in W_1 and W_2	$1.14 \pm 10^{\text{b}}$	$-2.19 \pm 0.90^{\text{b}}$	$-1.13 \pm 1.40^{\text{b}}$

No <i>E. coli</i> -GFP	20% W ₁ , 1% Tw80, no NaCl in W ₁ and W ₂	-0.97±0.40 ^b	0.44±2.40 ^b	-0.73±1.30 ^b
	20% W ₁ , 5% Tw80, 0.085 M NaCl in W ₁ only	9.6±2.6 ^c	11.70±1.00 ^c	21.80±0.30 ^d
	20% W ₁ , 5% Tw80, 0.085 M NaCl in W ₁ and W ₂	2.20±2.20 ^{ab}	-1.83±1.60 ^b	-2.70±1.60 ^b
	20% W ₁ , 5% Tw80, no NaCl in W ₁ and W ₂	-2.00±0.40 ^b	-0.95±0.60 ^b	-0.28±0.20 ^b
	40% W ₁ , 1% Tw80, 0.085 M NaCl in W ₁ only	22.60±1.30 ^d	42.8±2.50 ^e	50.94±2.60 ^f
	40% W ₁ , 1% Tw80, 0.085 M NaCl in W ₁ and W ₂	-0.60±0.30 ^b	-0.67±0.3 ^b	-0.65±0.80 ^b
	40% W ₁ , 1% Tw80, no NaCl in W ₁ and W ₂	1.15±0.50 ^{ab}	0.86±0.60 ^{ab}	0.61±0.50 ^b
	40% W ₁ , 5% Tw80, 0.085 M NaCl in W ₁ only	24.80±0.70 ^d	40.72±0.80 ^e	50.38±1.60 ^f
	40% W ₁ , 5% Tw80, 0.085 M NaCl in W ₁ and W ₂	0.08±0.60 ^b	-0.01±0.60 ^b	0.26±0.60 ^b
	40% W ₁ , 5% Tw80, no NaCl in W ₁ and W ₂	0.49±0.33 ^b	0.14±0.10 ^b	0.37±0.10 ^b
	20% W ₁ , 1% Tw80, 0.085 M NaCl in W ₁ only	7.86±1.00 ^{adehghj}	13.21±1.40 ^{gh}	24.76±3.00 ^f
	20% W ₁ , 1% Tw80, 0.085 M NaCl in W ₁ and W ₂	0.88±0.50 ^{bcdj}	0.49±0.50 ^{cj}	0.97±0.30 ^{cdj}
	20% W ₁ , 1% Tw80, no NaCl in W ₁ and W ₂	0.93±0.90 ^{bcdj}	-0.57±0.80 ^{cj}	2.00±0.40 ^{acdj}
	20% W ₁ , 5% Tw80, 0.085 M NaCl in W ₁ only	7.18±0.70 ^{deghj}	10.25±1.30 ^{hj}	21.11±1.20 ^f

<i>E. coli</i> -GFP	20% W ₁ , 5% Tw80, 0.085 M NaCl in W ₁ and W ₂	-0.36±1.00 ^{cj}	-0.11±0.90 ^{cj}	-0.11±0.70 ^c
	20% W ₁ , 5% Tw80, no NaCl in W ₁ and W ₂	1.95±0.70 ^{cej}	0.69±0.50 ^{cj}	1.11±0.90 ^{cdj}
	40% W ₁ , 1% Tw80, 0.085 M NaCl in W ₁ only	21.21±1.10 ^f	37.20±0.90 ⁱ	50.90±2.60 ^k
	40% W ₁ , 1% Tw80, 0.085 M NaCl in W ₁ and W ₂	-0.61±0.50 ^{cj}	-1.50±0.70 ^{cj}	-1.20±1.70 ^c
	40% W ₁ , 1% Tw80, no NaCl in W ₁ and W ₂	2.30±1.00 ^{cj}	2.10±1.30 ^{cej}	2.20±0.80 ^{cd}
	40% W ₁ , 5% Tw80, 0.085 M NaCl in W ₁ only	19.90±1.90 ^{fg}	35.90±2.00 ⁱ	47.90±2.00 ^k
	40% W ₁ , 5% Tw80, 0.085 M NaCl in W ₁ and W ₂	0.70±0.40 ^{cej}	-4.10±0.30 ^c	0.32±0.70 ^c
	40% W ₁ , 5% Tw80, no NaCl in W ₁ and W ₂	1.93±0.40 ^{cej}	4.30±0.90 ^j	0.29±1.10 ^c

The data was analysed with one-way ANOVA

a, b, c, d, e, f, g, h, i, j, k means ± standard deviation with different letters are significantly different

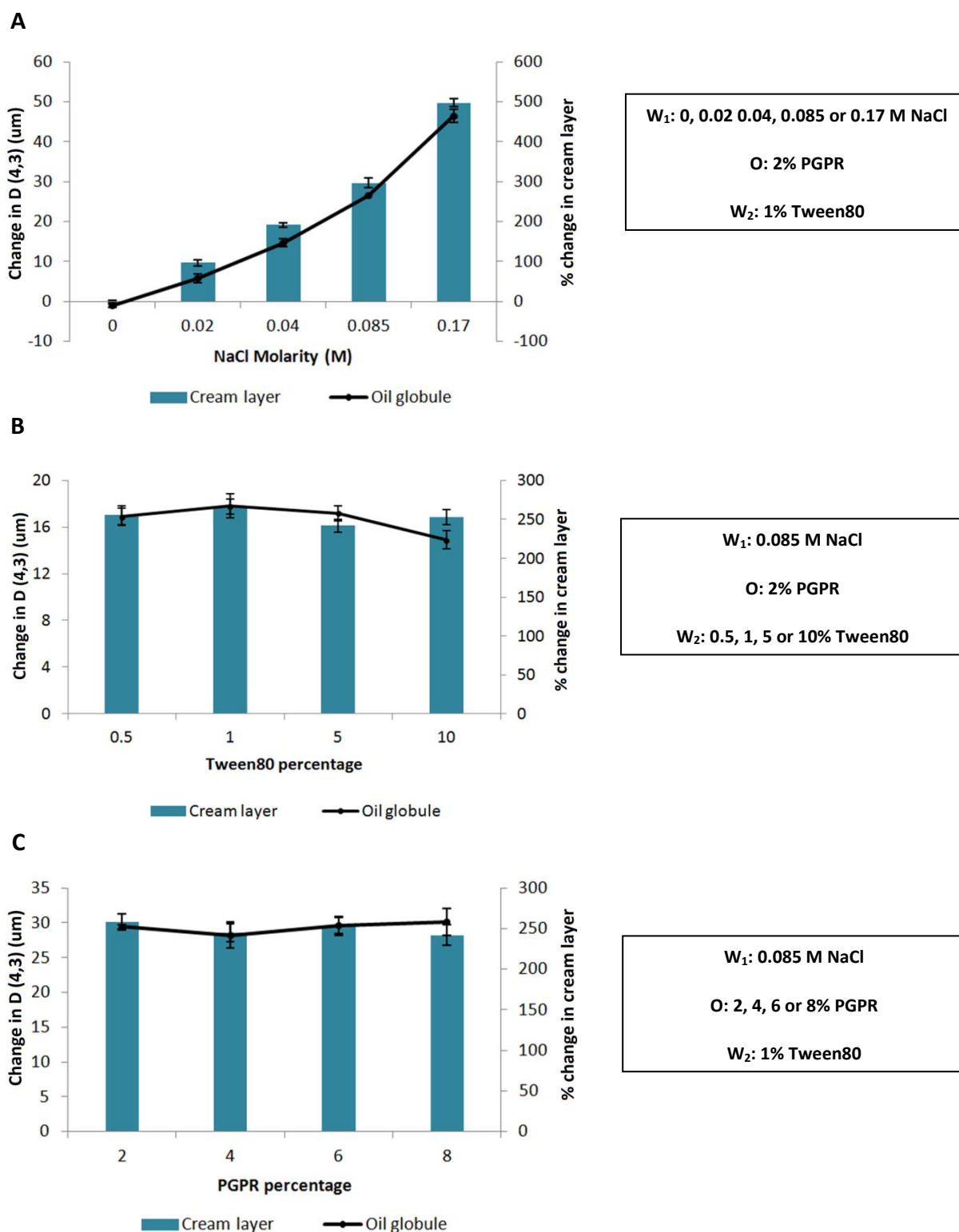


Figure 6.3 Change in mean oil globule diameter size (μm) by light scattering [$D(4, 3)$] and percentage in cream layer thickness of $W_1/O/W_2$ emulsions in hypo-osmotic solution after 45-minutes relative to time 0 minutes at 25°C . The $W_1/O/W_2$ emulsions were prepared: A) 40% W_1 containing *E. coli*-GFP and varying concentrations of NaCl (0.02 M, 0.04 M, 0.085 M or 0.17 M) in W_1 and stabilised with 1% Tween80 in W_2 and 2% PGPR; B) 40% W_1 containing *E. coli*-GFP and 0.085 M NaCl in W_1 and and stabilised with varying concentrations of Tween80 (0.5, 1, 5 or 10%) in W_2 and 2% PGPR in the oil phase; C) 40% W_1

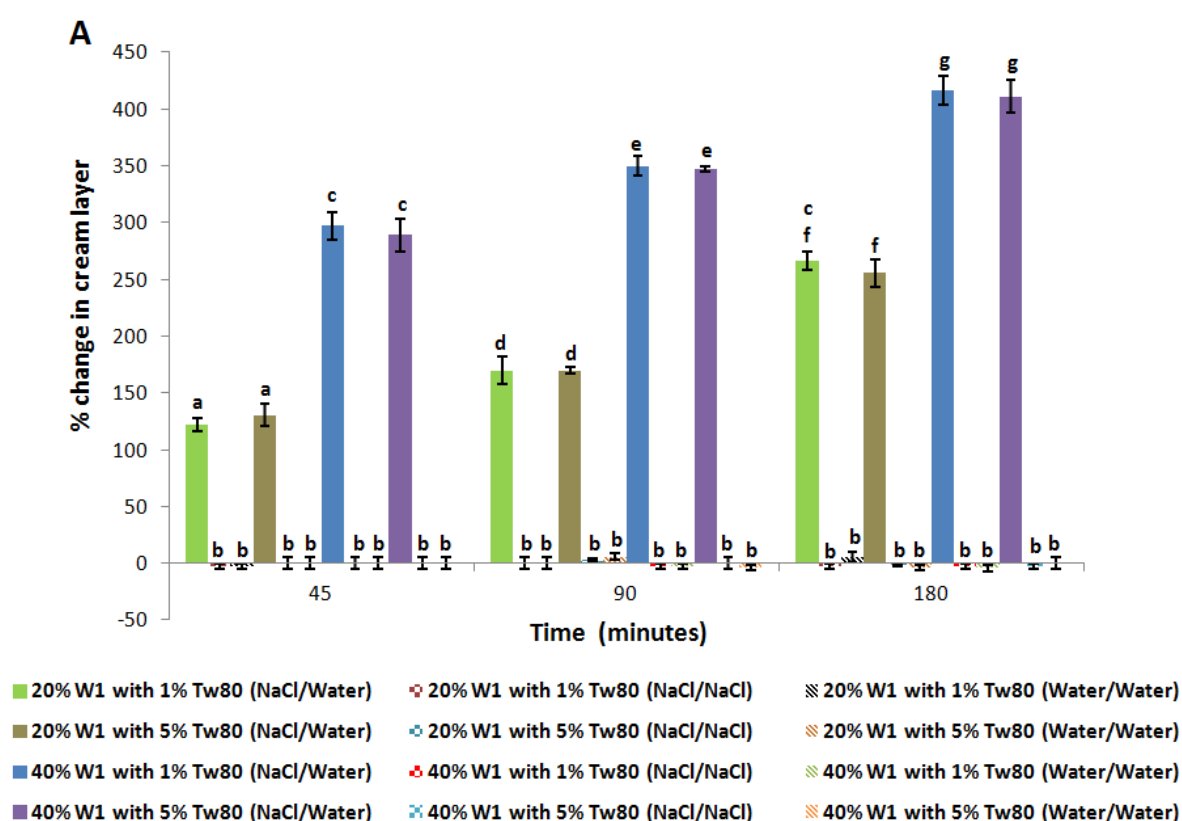
containing *E. coli*-GFP and 0.085 M NaCl in W_1 and stabilised with 1% Tween80 in W_2 and varying concentrations of PGPR (2, 4, 6 or 8%) in the oil phase. Results are taken from a minimum of 3 independent experiments. The data was analysed with one-way ANOVA.

6.3.5 Changes in creaming behaviour during osmotic balance alteration and storage

Percentage gain in cream layer thickness over time was measured to characterise the creaming behaviour of $W_1/O/W_2$ emulsions containing *E. coli*-GFP in W_1 as an effect of varying the osmotic pressure gradient (Figure 5.3A), concentration of Tween80 (Figure 5.3B), and PGPR (Figure 5.3C). The effect of varying the concentration of W_1 and Tween80 on the percentage gain in cream layer thickness of the $W_1/O/W_2$ emulsions with and without altering the osmotic balance in the presence or absence of *E. coli*-GFP in W_1 (Figure 5.4A and B) was measured.

The presence of bacteria in W_1 had no effect on the cream layer thickness during the incubation period (Figures 5.4A and B). The percentage gain in cream layer thickness was significantly ($P<0.05$) increased over time when the osmotic balance was altered compared to when unaltered (Figures 5.3A, 5.4A and B). With the formulation containing 40% W_1 and stabilised with 1% Tween80 and during osmotic balance alteration the gain in cream layer thickness was significantly ($P<0.05$) increased when the concentration of NaCl in W_1 was high (0.085 M and 0.17 M) compared to when the concentration of NaCl in W_1 was low (0.02 M and 0.04 M) (Figure 5.3A). During osmotic balance alteration, there was no significant change in cream layer thickness at different concentrations of Tween80 (Figure 5.3B) or PGPR (Figure 5.3C). During no osmotic balance alteration and regardless of W_1 and Tween80 concentration there were no significant changes in cream layer thickness

(Figures 5.4A and B). During osmotic balance alteration, the gain in cream layer thickness was significantly ($P<0.05$) higher at 40% W_1 compared to 20% W_1 regardless of Tween80 concentration (Figures 5.4A and B). These results indicate that creaming behaviour is affected by the concentration of NaCl and W_1 during osmotic balance alteration whilst the concentration of Tween80 and PGPR had no effect.



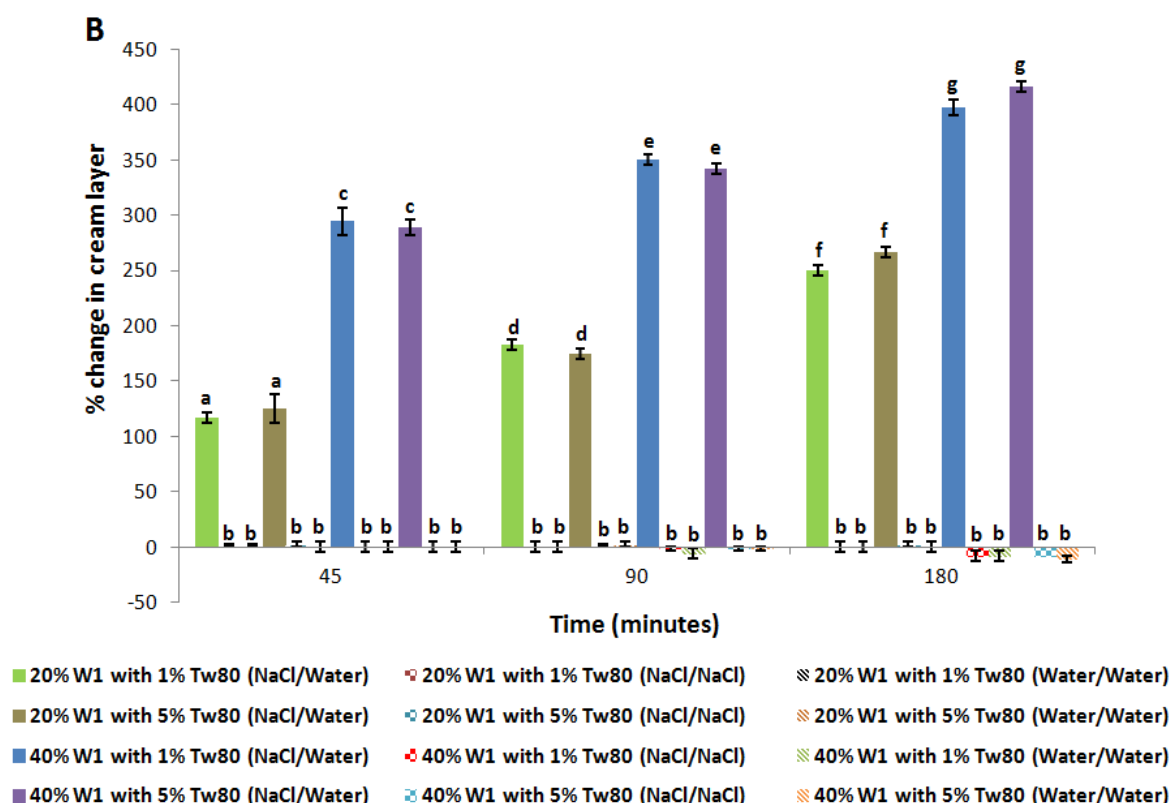


Figure 6.4 Change of percentage in cream layer thickness of $W_1/O/W_2$ emulsions after 45, 90 and 180-minutes relative to time 0 minutes incubated at 25°C. The $W_1/O/W_2$ emulsions were prepared with different inner-phase (W_1) volume percentage of 20% or 40% with (A) or without (B) *E. coli*-GFP with or without 0.085 M NaCl in the W_1 phase stabilised with 1% or 5% Tween80 in W_2 and 2% PGPR in the oil phase. Bars represent mean \pm SEM taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different ($P < 0.05$). The data was analysed with one-way ANOVA.

6.3.6 Microscopic observation of oil globule swelling, bursting and *E. coli*-GFP release

The $W_1/O/W_2$ emulsions were observed using fluorescence microscopy to investigate the effect of altering the osmotic balance and changing the concentration of W_1 and Tween80 on the structure of the $W_1/O/W_2$ emulsion and the release of *E. coli*-GFP from W_1 to W_2 . After osmotic balance alteration, an increase in size of the $W_1/O/W_2$ globules was observed as well as the presence of *E. coli*-GFP in both W_1 and W_2 phases. During osmotic balance alteration and at 40% and 1% (Figure 6.7C)

or 5% (Figure 6.8C) Tween80 the oil globules showed swelling and *E. coli*-GFP cells were present in the W_1 phase and in the W_2 phase (Figure 6.11C and 6.12C). During osmotic balance alteration and at 20% W_1 and 1% (Figure 6.5C) or 5% (Figure 6.6C) Tween80 the oil globules showed swelling whilst *E. coli*-GFP cells were mainly present in the W_1 phase with very few present in the W_2 phase (Figure 6.9C and 6.10C). During no osmotic balance alteration, the oil globules showed no swelling (Figure 6.5-6.8) whilst *E. coli*-GFP cells mainly remained within the W_1 phase (Figure 6.9-6.12).

To confirm that the increase in D (4, 3) of oil globules in hypo-osmotic solution was due to water movement from W_2 to W_1 and not solely because of coalescence between the oil globules the evolution of a single oil globule was followed under hypo-osmotic conditions. Figure 6.13 shows snapshots of an oil globule taken after diluting the $W_1/O/W_2$ emulsion made with 40% W_1 containing NaCl and stabilised with 8% PGPR and 1% Tween80 in a hypo-osmotic solution. The oil globule showed a progressive increase in diameter over time with no observed coalescence confirming that swelling of the oil globule is occurring because of water movement from W_2 to W_1 . The swelling phenomenon was a reproducible observation and occurred for all the oil globules under hypo-osmotic pressure.

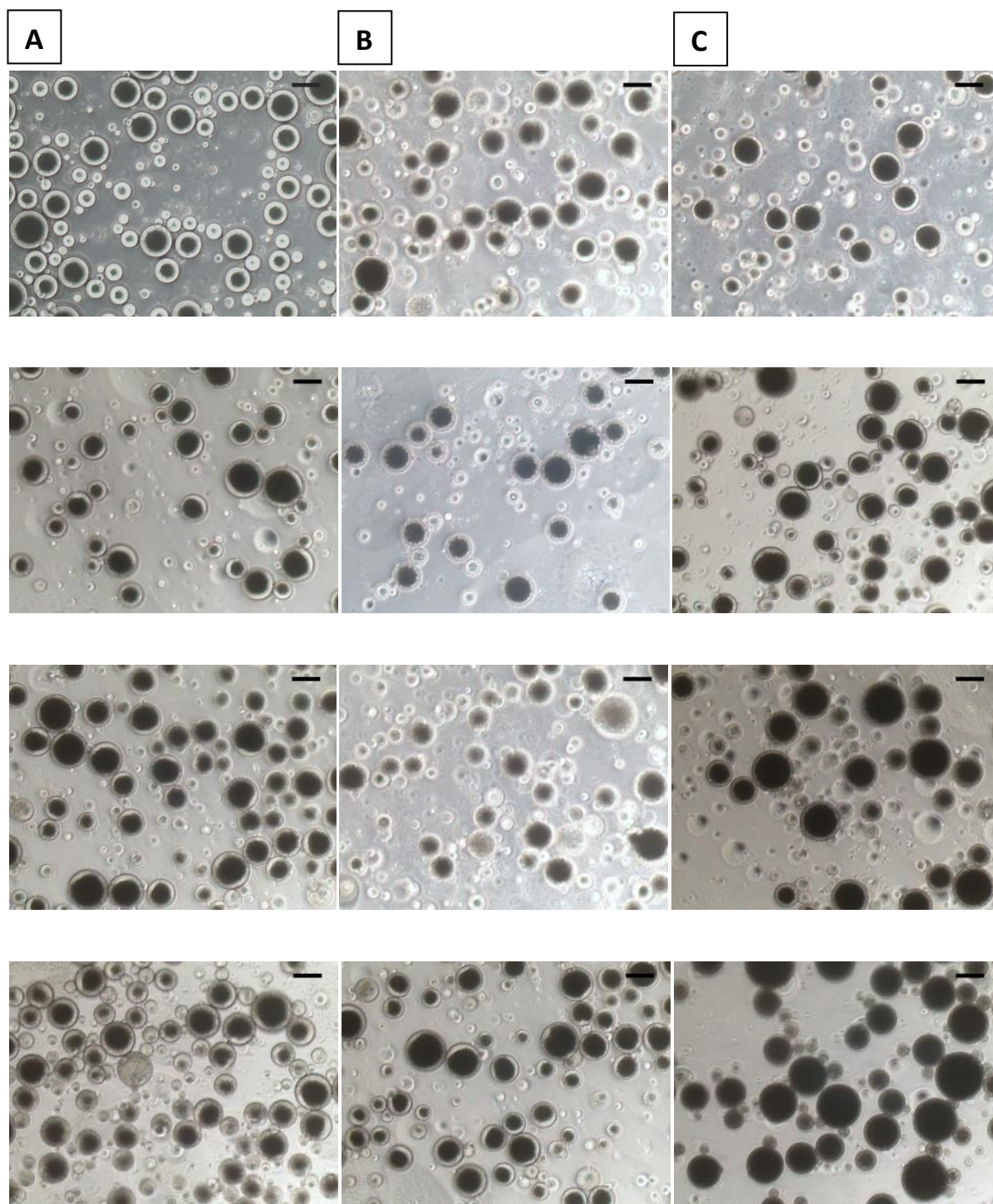


Figure 6.5 Optical microscopy images of $W_1/O/W_2$ double emulsions at 0, 2, 4 and 6 hours (from the top). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 20% containing *E. coli*-GFP with or without 0.085 M NaCl in the W_1 phase and stabilised with 1% Tween80 in W_2 and 2% PGPR in the oil phase. The formulations were made with no NaCl in W_1 or W_2 (A), 0.085 M NaCl in W_1 and W_2 (B) and 0.085 M NaCl in W_1 only (C). Scale bar: 50 μ m.

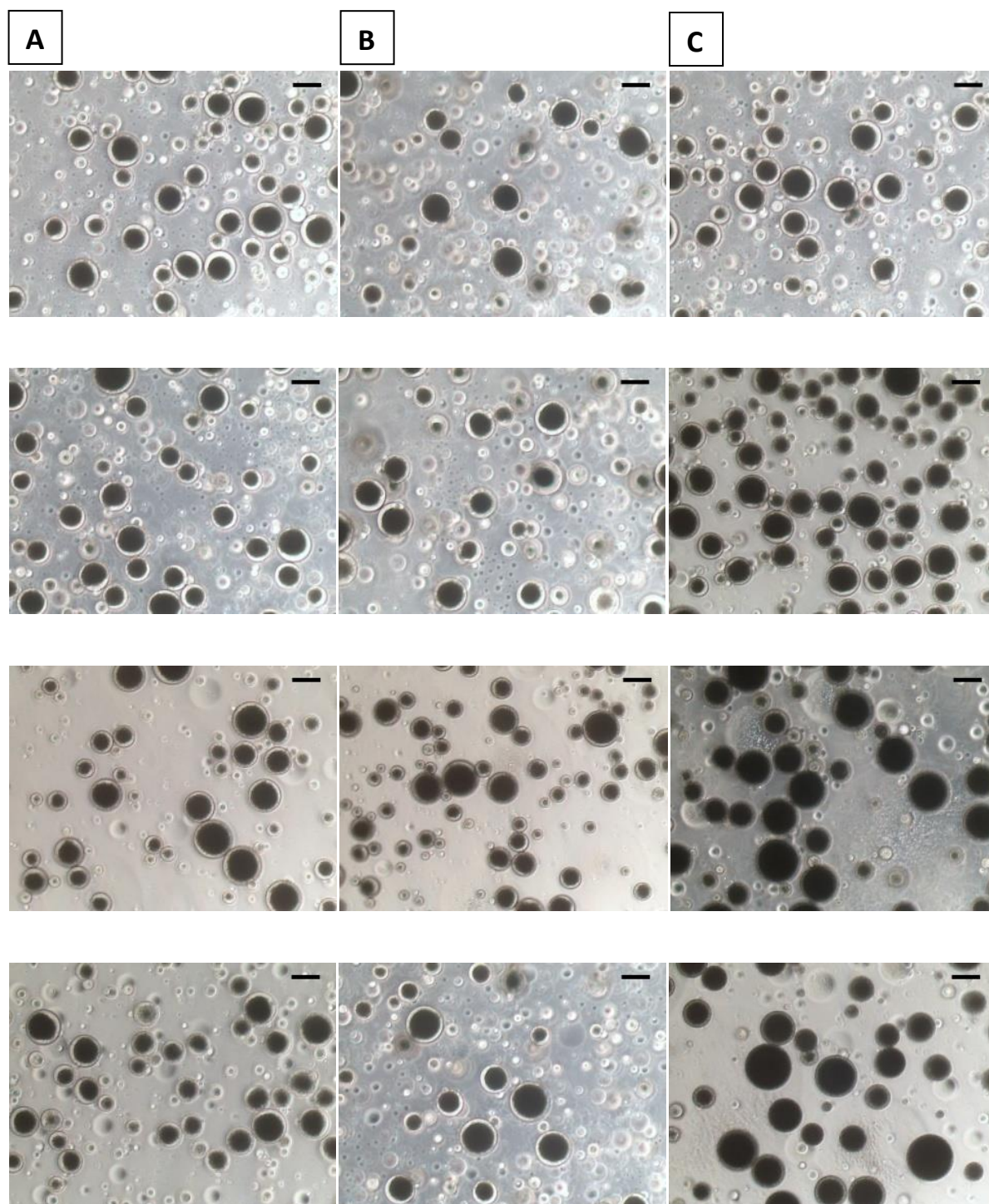


Figure 6.6 Optical microscopy images of $W_1/O/W_2$ double emulsions at 0, 2, 4 and 6 hours (from the top). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 20% containing *E. coli*-GFP with or without 0.085 M NaCl in the W_1 phase and stabilised with 5% Tween80 in W_2 and 2% PGPR in the oil phase. The formulations were made with no NaCl in W_1 or W_2 (A), 0.085 M NaCl in W_1 and W_2 (B) and 0.085 M NaCl in W_1 only (C). Scale bar: 50 μ m.

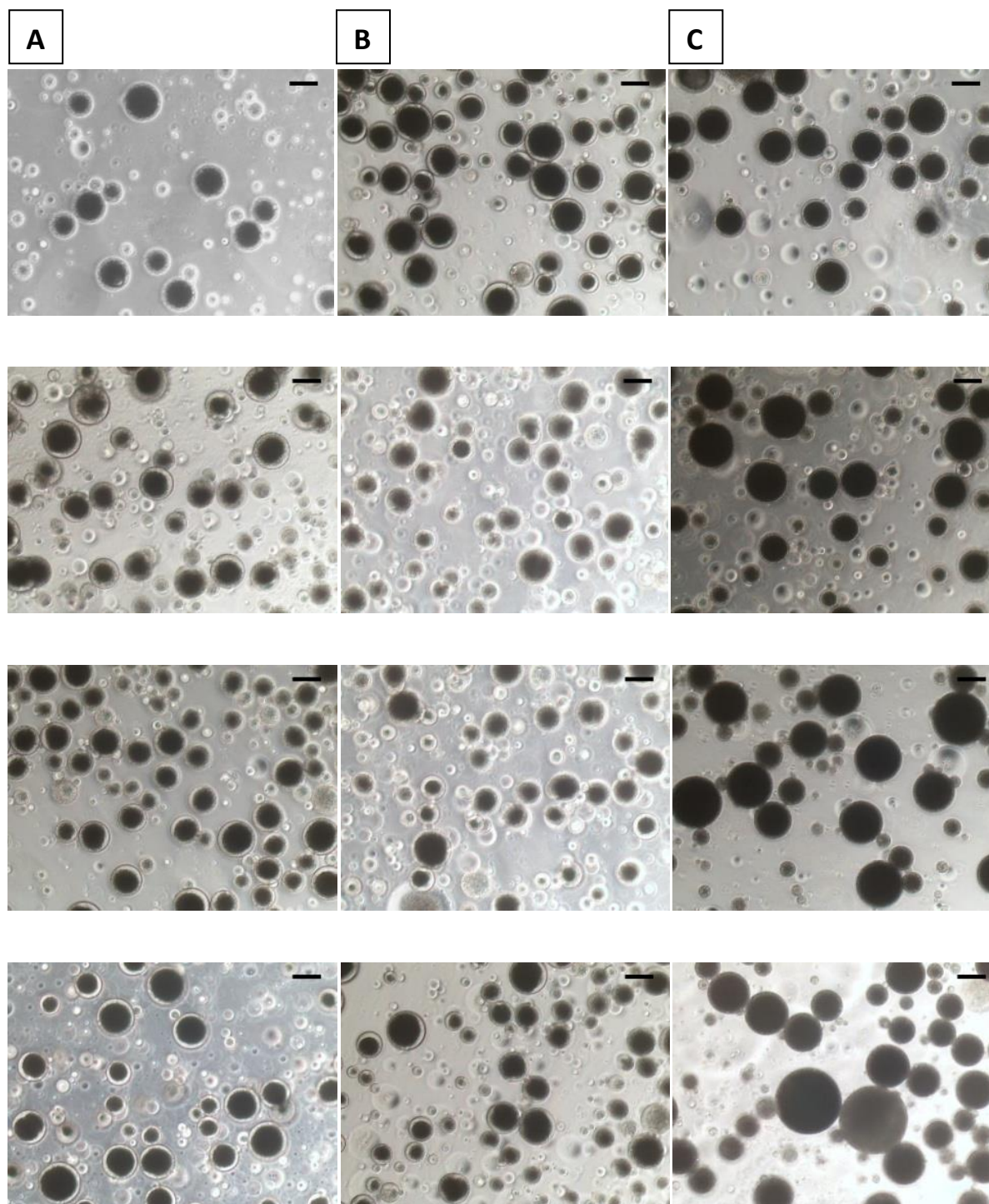


Figure 6.7 Optical microscopy images of $W_1/O/W_2$ double emulsions at 0, 2, 4 and 6 hours (from the top). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 40% containing *E. coli*-GFP with or without 0.085 M NaCl in the W_1 phase and stabilised with 1% Tween80 in W_2 and 2% PGPR in the oil phase. The formulations were made with no NaCl in W_1 or W_2 (A), 0.085 M NaCl in W_1 and W_2 (B) and 0.085 M NaCl in W_1 only (C). Scale bar: 50 μ m.

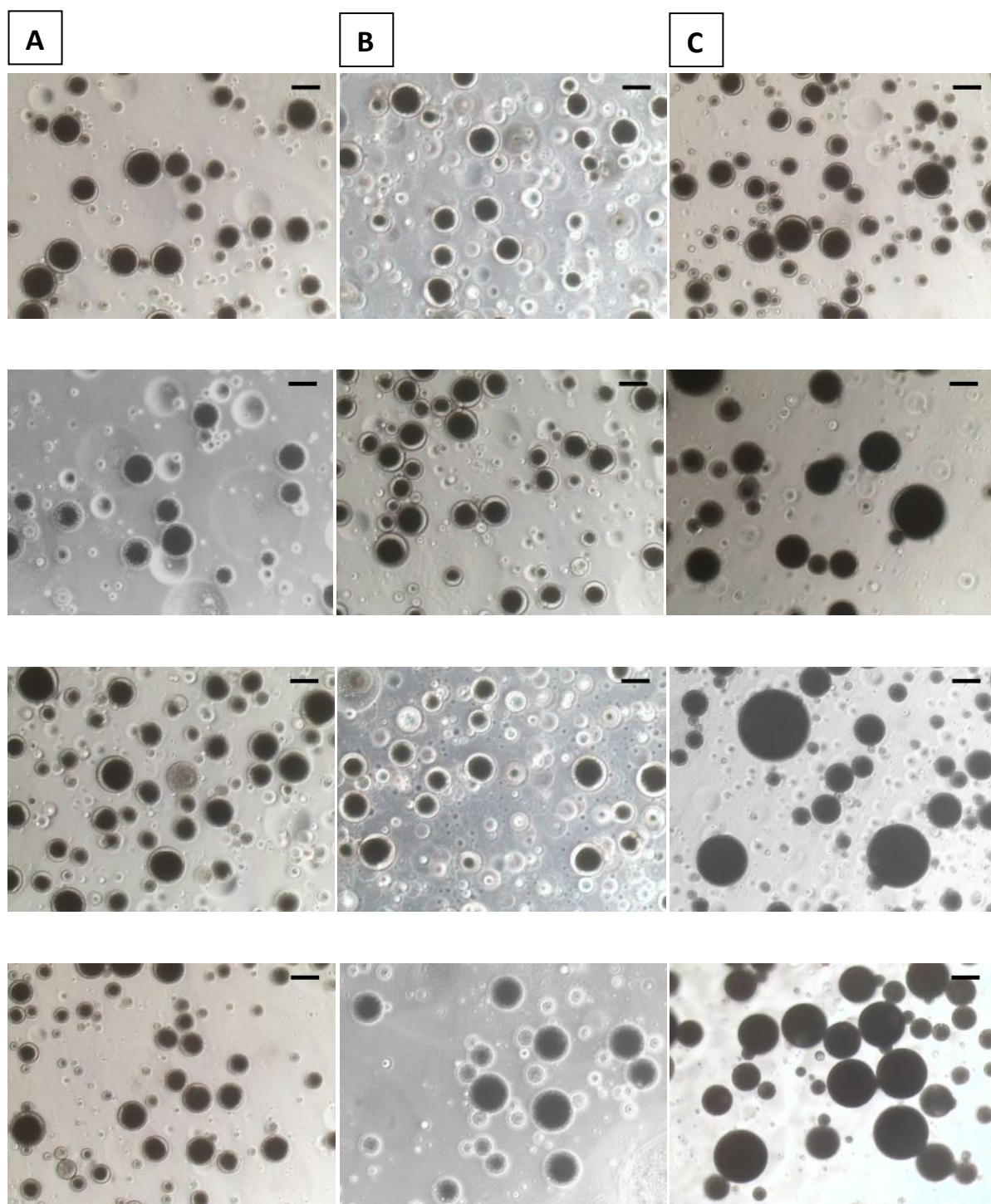


Figure 6.8 Optical microscopy images of $W_1/O/W_2$ double emulsions at 0, 2, 4 and 6 hours (from the top). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 40% containing *E. coli*-GFP with or without 0.085 M NaCl in the W_1 phase and stabilised with 5% Tween80 in W_2 and 2% PGPR in the oil phase. The formulations were made with no NaCl in W_1 or W_2 (A), 0.085 M NaCl in W_1 and W_2 (B) and 0.085 M NaCl in W_1 only (C). Scale bar: 50 μ m.

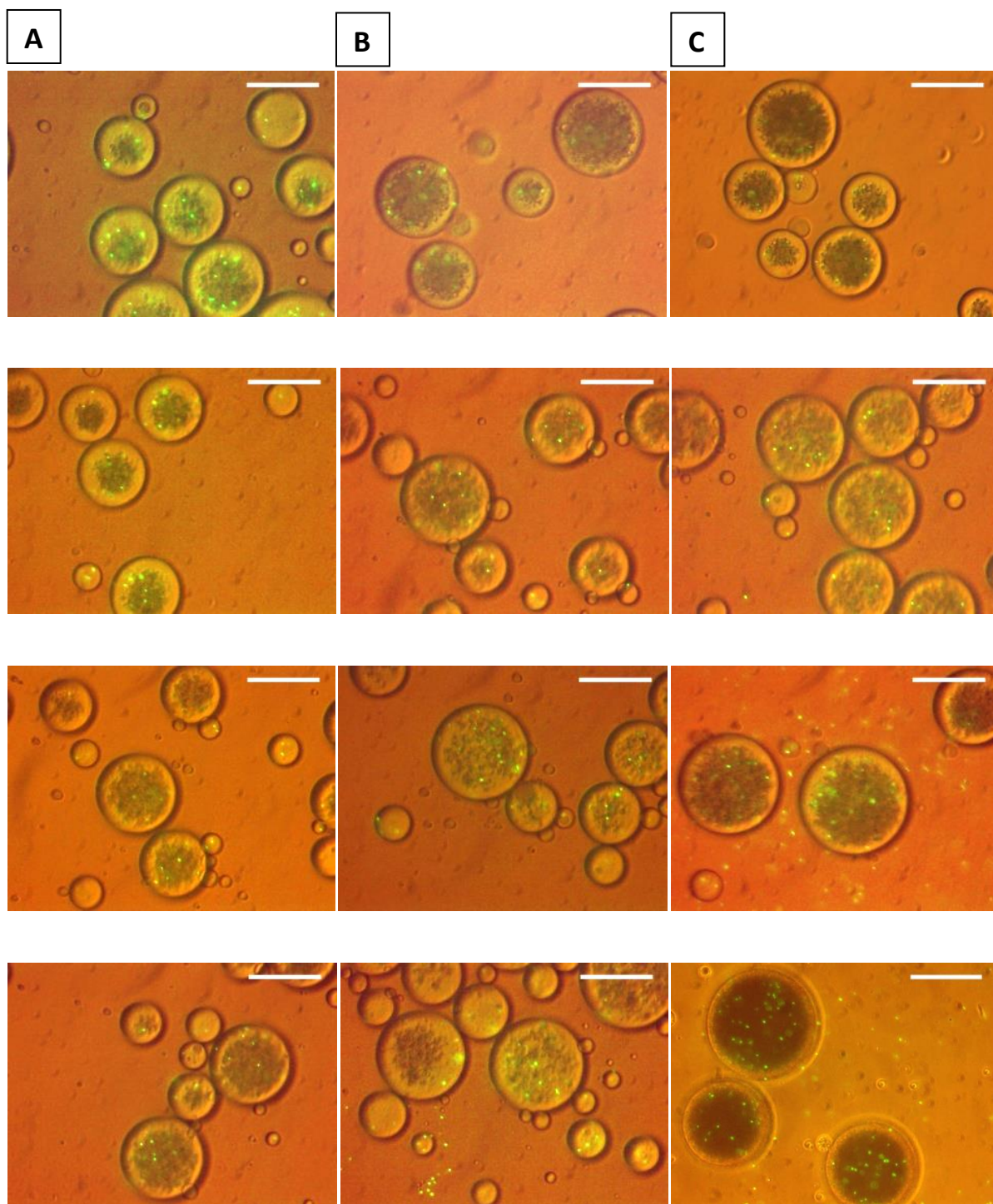


Figure 6.9 Photomicrographs composed from the optical and fluorescence images of *E. coli*-GFP within $W_1/O/W_2$ double emulsions at 0, 2, 4 and 6 hours (from the top). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 20% containing *E. coli*-GFP with or without 0.085 M NaCl in the W_1 phase and stabilised with 1% Tween80 in W_2 and 2% PGPR in the oil phase. The formulations were made with no NaCl in W_1 or W_2 (A), 0.085 M NaCl in W_1 and W_2 (B) and 0.085 M NaCl in W_1 only (C). Scale bar: 100 μ m.

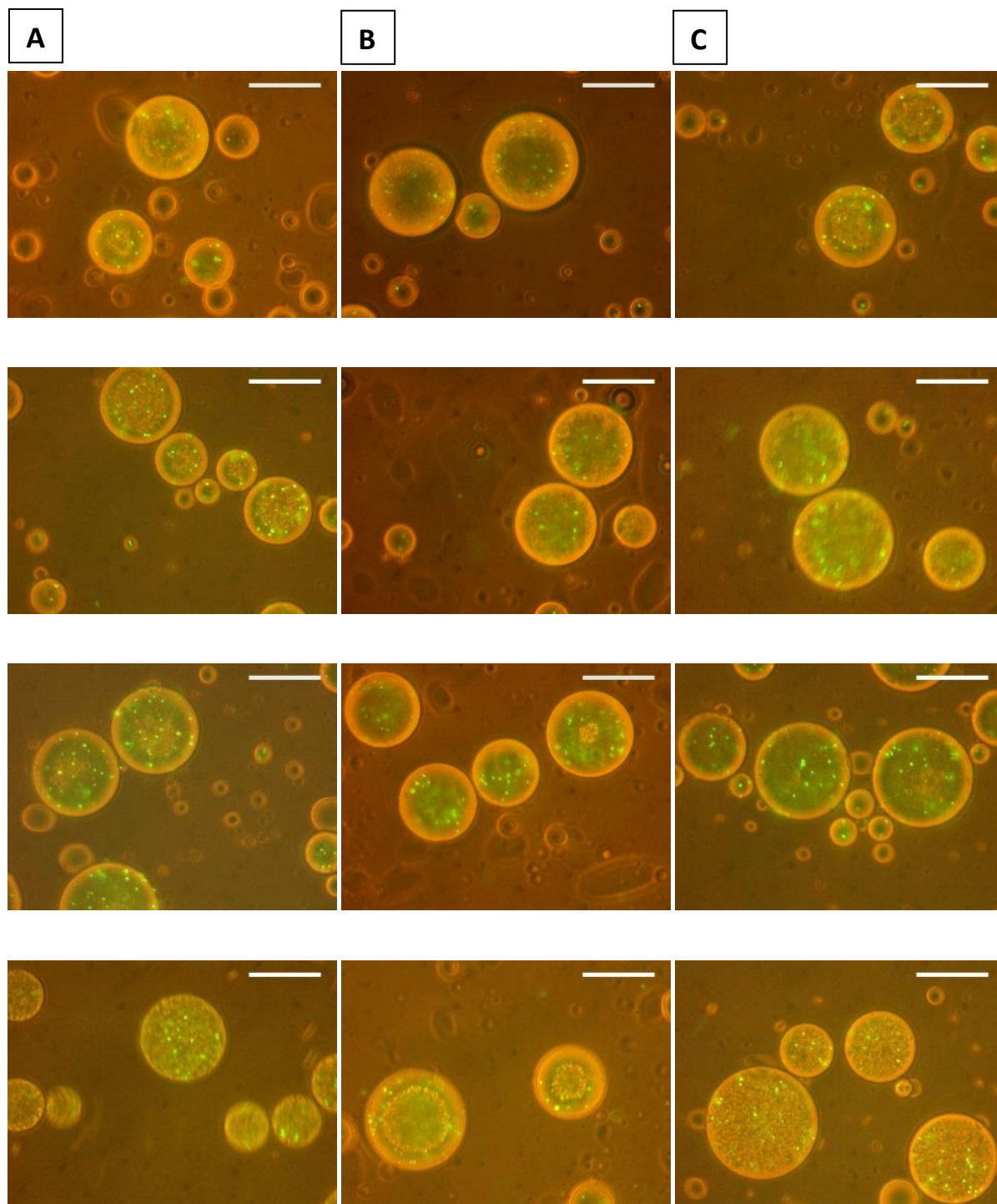


Figure 6.10 Photomicrographs composed from the optical and fluorescence images of *E. coli*-GFP within $W_1/O/W_2$ double emulsions at 0, 2, 4 and 6 hours (from the top). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 20% containing *E. coli*-GFP with or without 0.085 M NaCl in the W_1 phase and stabilised with 5% Tween80 in W_2 and 2% PGPR in the oil phase. The formulations were made with no NaCl in W_1 or W_2 (A), 0.085 M NaCl in W_1 and W_2 (B) and 0.085 M NaCl in W_1 only (C). Scale bar: 100 μ m.

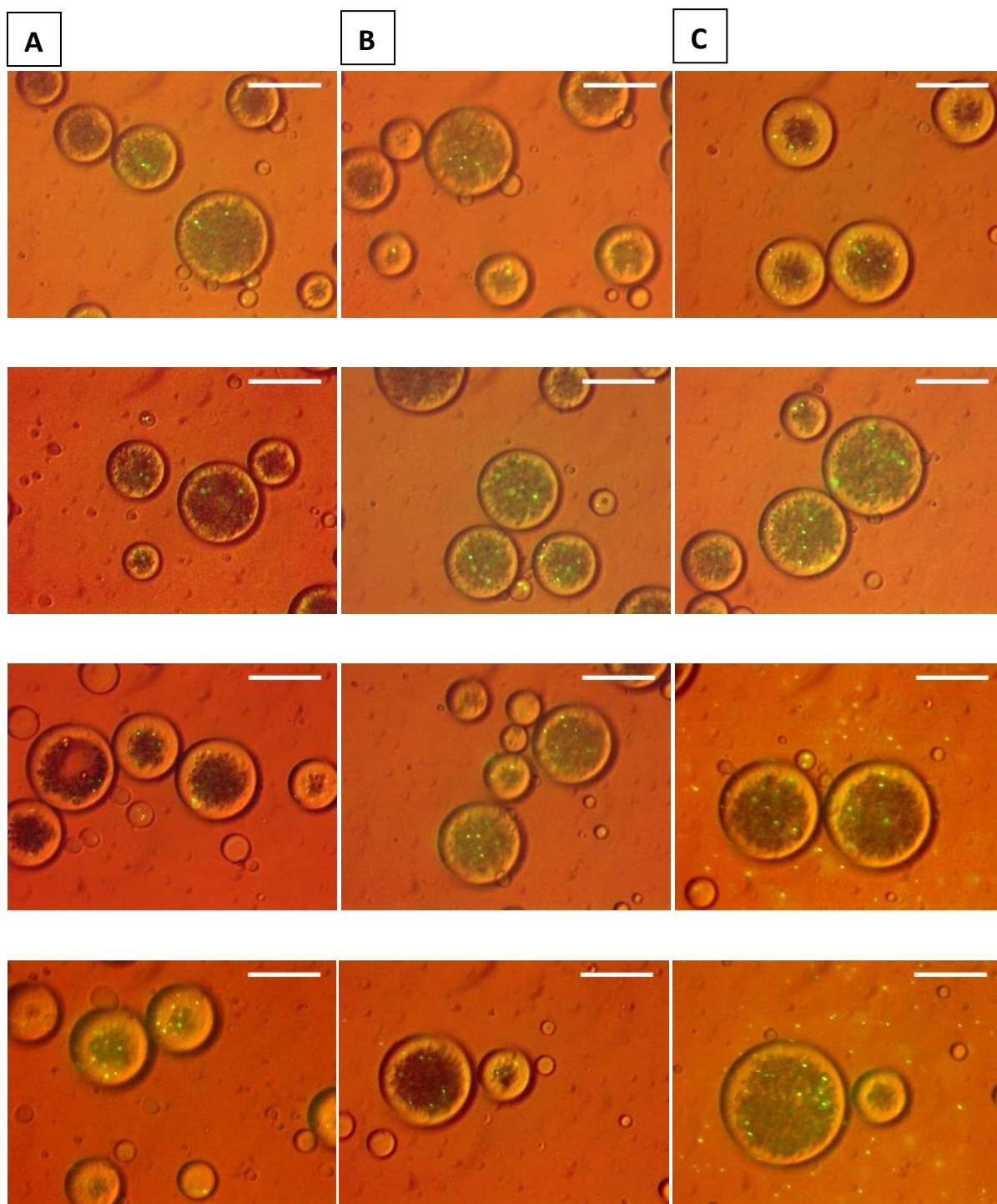


Figure 6.11 Photomicrographs composed from the optical and fluorescence images of *E. coli*-GFP within $W_1/O/W_2$ double emulsions at 0, 2, 4 and 6 hours (from the top). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 40% containing *E. coli*-GFP with or without 0.085 M NaCl in the W_1 phase and stabilised with 1% Tween80 in W_2 and 2% PGPR in the oil phase. The formulations were made with no NaCl in W_1 or W_2 (A), 0.085 M NaCl in W_1 and W_2 (B) and 0.085 M NaCl in W_1 only (C). Scale bar: 100 μ m.

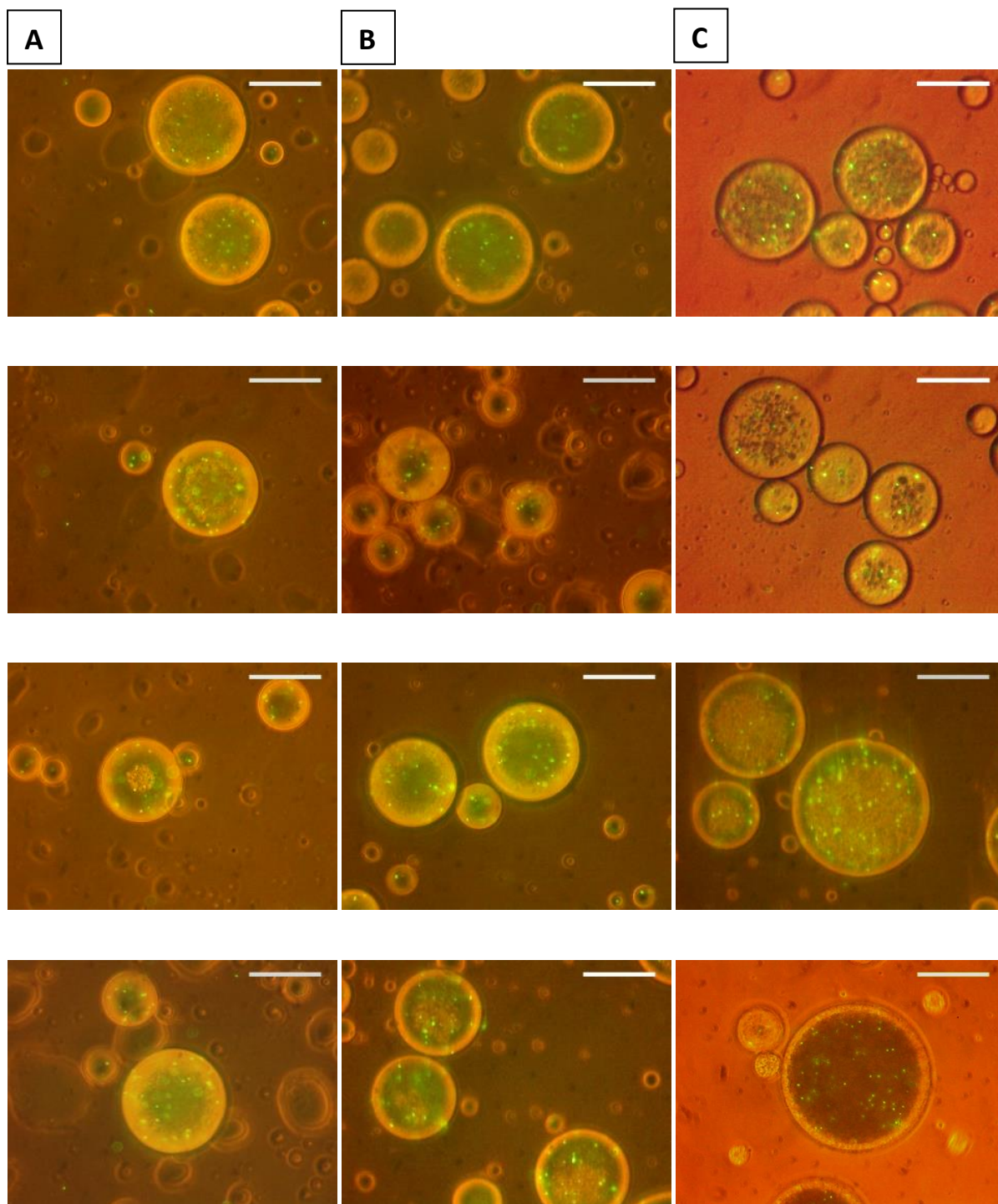


Figure 6.12 Photomicrographs composed from the optical and fluorescence images of *E. coli*-GFP within $W_1/O/W_2$ double emulsions at 0, 2, 4 and 6 hours (from the top). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 40% containing *E. coli*-GFP with or without 0.085 M NaCl in the W_1 phase and stabilised with 5% Tween80 in W_2 and 2% PGPR in the oil phase. The formulations were made with no NaCl in W_1 or W_2 (A), 0.085 M NaCl in W_1 and W_2 (B) and 0.085 M NaCl in W_1 only (C). Scale bar: 100 μ m.

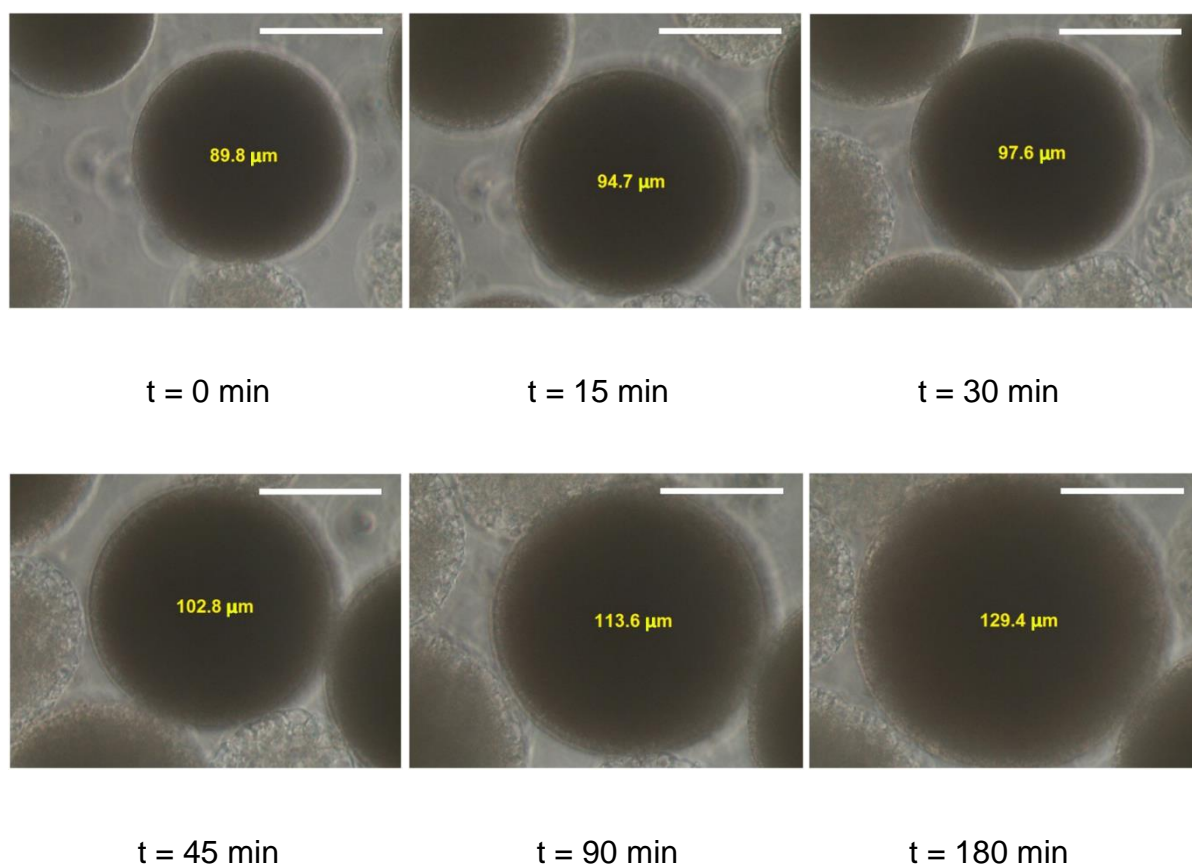


Figure 6.13 Optical snap shot images showing one $W_1/O/W_2$ emulsion oil globule swelling in hypo-osmotic solution over time (0, 15, 30, 45, 90 and 180 minutes). The $W_1/O/W_2$ emulsions were prepared with inner-phase (W_1) volume percentage of 40% containing *E. coli*-GFP with 0.085 M NaCl in the W_1 phase and stabilised with 1% Tween80 in W_2 and 8% PGPR in the oil phase. To alter the osmotic balance the $W_1/O/W_2$ globules were injected into a fresh W_2 phase with 1% Tween80 containing no salt. The diameter of the oil globule (μm) over time is shown in yellow. Scale bar: 50μm.

So far, our results showed that the release of *E. coli*-GFP is significantly increased during osmotic balance alteration by increasing the amount of W_1 and decreasing the concentration of Tween80 or PGPR. Therefore, further investigation was carried out to observe the mechanism of bacterial release using fluorescence video microscopy. Using video-microscopy we could observe the bursting mechanism responsible for the release of *E. coli*-GFP from the oil globules after diluting the

$W_1/O/W_2$ emulsion in a hypo-osmotic solution. Figure 6.14 shows snapshots taken after diluting the $W_1/O/W_2$ emulsion made with 40% W_1 containing NaCl and stabilised with 2% PGPR and 1% Tween80 in a hypo-osmotic solution (Appendix, Video A5). The interfacial film of the oil globule ruptures and W_1 droplets with *E. coli*-GFP cells are released into the W_2 phase and the oil phase no longer form a droplet.

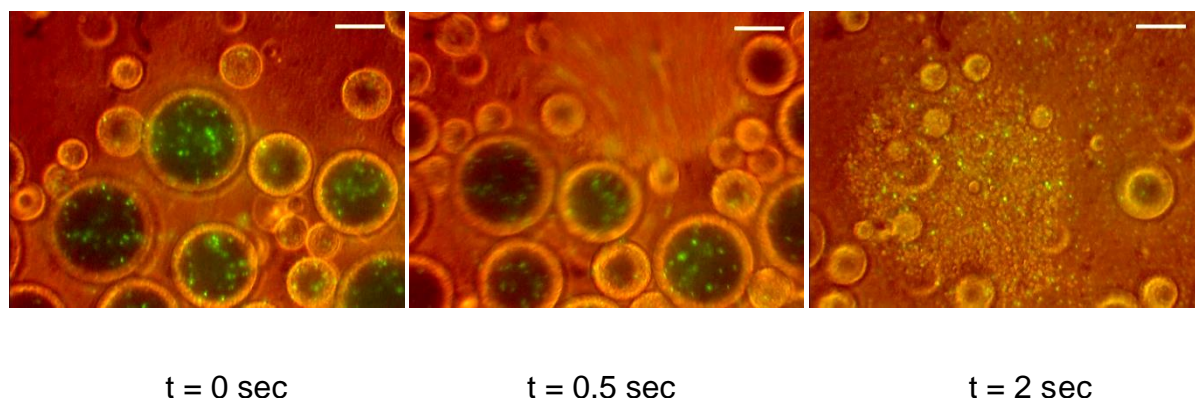


Figure 6.14 Fluorescence snap shot images obtained from video-microscopy showing oil globule bursting and release of W_1 droplets and *E. coli*-GFP after osmotic balance alteration. Fluorescent images of burst release of *E. coli*-GFP (green) from an oil globule (arrows) in a $W_1/O/W_2$ emulsion at different time points. The $W_1/O/W_2$ emulsion was prepared with inner W_1 phase volume percentage of 40% containing 0.085 M NaCl with bacteria and stabilised with 1% Tween80 in W_2 and 2% PGPR in the oil phase. Scale bar: 50 μ m.

6.3.7 Effect of encapsulation and release on *E. coli* viability

The effects of $W_1/O/W_2$ emulsion structure and the release mechanism on *E. coli* was studied by measuring the viability of bacteria by culture on media and microscopic assessment of bacterial cells stained for injury. There was no significant difference in the number of *E. coli* cells obtained from the plating method compared to microscopic enumeration (Figure 6.15). Also after release into the W_2 phase, there were no DiBAC₄ (3) positive *E. coli* cells (Figure 6.16) and since DiBAC₄ (3) can enter depolarised cells this suggests that encapsulation and release had no effect on *E. coli* membrane potential.

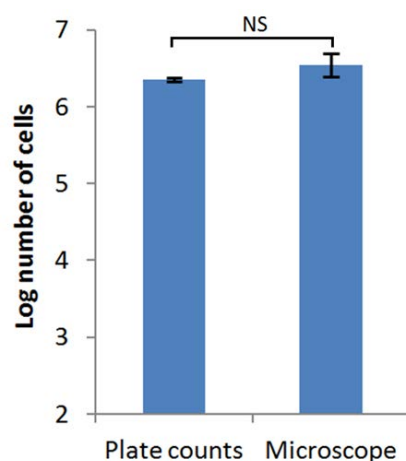


Figure 6.15 Log number of released *E. coli* cells in serum phase quantified by plate counting and microscopic enumeration after 180-minute incubation at 25°C. The $W_1/O/W_2$ emulsion was made with 40% W_1 with *E. coli* with 0.085 M NaCl in the W_1 phase stabilised with 1% Tween80 in W_2 and 2% PGPR in the oil phase. Bars represent mean \pm SEM taken from a minimum of 3 independent experiments. The data was analysed with Student's *t*-Test.

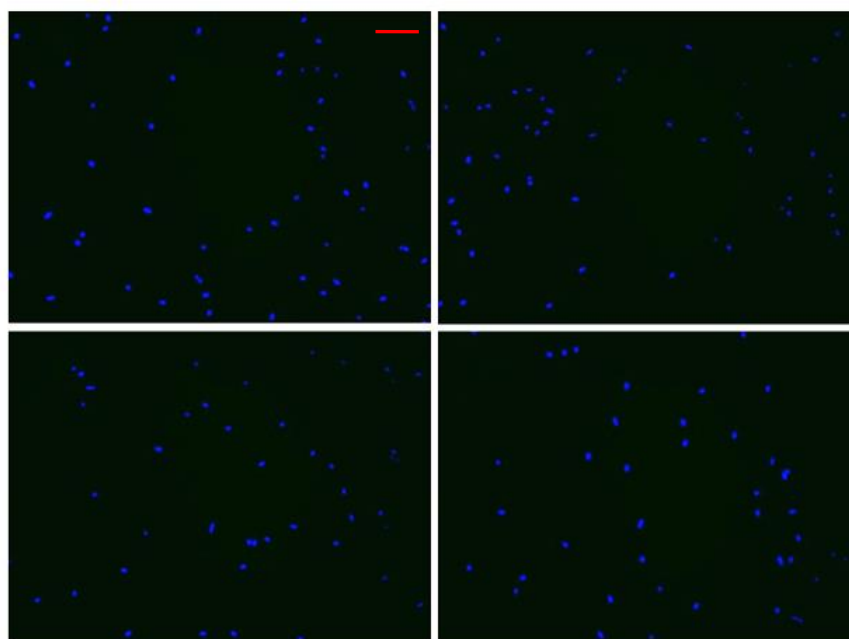


Figure 6.16 Photomicrographs composed from the optical and fluorescence images of released *E. coli* cells in serum phase (right) after 180 minutes stained with the DNA stain DAPI and membrane potential-sensing stain DiBAC₄ (3). The $W_1/O/W_2$ emulsion was made with 40% W_1 with *E. coli* with 0.085 M NaCl in the W_1 phase stabilised with 1% Tween80 in W_2 and 2% PGPR in the oil phase. Scale bar: 10 μ m.

6.4 Discussion

In agreement with previous studies (Wen and Papadopoulos, 2001; Jager-Lezer, 1997; Sapei *et al.*, 2012) it was found that when the $W_1/O/W_2$ emulsion was diluted in a hypo-osmotic solution the oil globules became larger in size. As water migrates from W_2 to W_1 the swelling of the oil globules diminishes because of the reduction in the concentration gradient. The swelling of the oil globules due to the presence of NaCl in the W_2 phase was shown to be concentration dependent as the oil globules were larger at higher concentrations (0.085 M and 0.17 M) compared to lower concentrations of NaCl (0.04 M and 0.02 M). These findings correlate with a study by Chong-Kook *et al.* concluding that the oil layer of $W_1/O/W_2$ emulsion globules acts as a water-permeable membrane between the two aqueous phases of the $W_1/O/W_2$ emulsion under the osmotic pressure gradient (Chong-Kook *et al.*, 2005). This phenomenon can be related to the osmotic pressure gradient created between the W_2 and W_1 phases during osmotic balance alteration (Cárdenas and Castro, 2003). It has been well demonstrated that water migration in $W_1/O/W_2$ emulsions occurs due to interfacial processes control the transport of water in $W_1/O/W_2$ emulsions rather than bulk diffusion (Jager-Lezer *et al.*, 1997). When the W_1 droplets and oil globule interfaces are not physically in contact mechanisms that control the transport of water between W_1 and W_2 phases include spontaneous emulsification and reverse micellisation (Wen and Papadopoulos, 2001). Another mechanism controls water transport when the W_1 and oil globule interfaces are physically in contact and that involves the hydration of the surfactant molecule at one interface, its diffusion through the oil phase to dehydrate at the other interface that is in contact with the phase of higher solute concentration (Wen and Papadopoulos, 2000a). In this study,

it was found that the concentrations of Tween80 (in the W_2 phase) or PGPR (in the oil phase) had no significant effect on the oil globule size.

In the video-microscopy results it was observed that after altering the osmotic balance with the formulation containing low concentration of Tween80 and high amount of the W_1 phase the oil globule bursts releasing W_1 droplets and *E. coli*-GFP into W_2 . It was also observed that the W_1 droplets persisted within the W_2 phase upon release. In the previous Chapter, the same observation was reported. However; more investigation is needed to understand the nature of these W_1 droplets. In systems where the osmotic pressure was unaltered minimal release of *E. coli*-GFP was observed probably due to mechanical breakdown (i.e. mixing) of the $W_1/O/W_2$ emulsion globules. The release of *E. coli*-GFP was higher when osmotic balance was altered compared to when unaltered which was NaCl concentration-dependent. During osmotic balance alteration and at low concentration of Tween80 release of *E. coli* cells from $W_1/O/W_2$ globules was increased regardless of W_1 concentration. Jager-Lezer *et al.* (1997) found that when $W_1/O/W_2$ emulsions were diluted in a hypo-osmotic solution the W_1 droplets increased in size leading to swelling of the oil globules until a critical size is reached. Moreover, the authors claim that beyond the critical size the oil globules will burst and release their inner contents into W_2 .

The mechanism of interfacial film rupture is determined by the characteristics of the continuous phase (e.g. viscosity) and the interfacial layers surrounding the oil globules (e.g. thickness, interfacial tension) (Shuster, 1985; Frising *et al.*, 2006). Above a certain critical film thickness, film rupture of the oil globule is improbable (Krebs *et al.*, 2012). However, film rupturing becomes more probable once the film has thinned to the critical thickness (Krebs *et al.*, 2012). The thinner the film the

lower the energy of activation required to form a hole that grows leading to film rupture and oil globule bursting (Shuster, 1985). Tween80 molecules can keep the oil globules apart as well as provide a barrier against rupture. When present at higher concentrations in the W_2 phase, hydrophilic surfactants tend to form condensed interfacial films (Tadros, 2013) resulting in an increased interfacial film strength (Jager-Lezer *et al.*, 1997). When Tween80 is present in W_2 at high amounts it adsorbs at the O/W_2 interface creating more condensed interfacial films which can resist hole creation and propagation that leads to film rupture (Nikiforidis and Kiosseoglou, 2011). Gaps that are formed at the O/W_2 interface due to incomplete surface coverage of the interface by the surfactant molecules or due to uneven distribution of surfactant molecules on the interface will lead to hole formation (McClements, 2015). If there are insufficient surfactant molecules in the system to saturate the O/W_1 interface there will be gaps in the interfacial film (McClements, 2015). Also during swelling the interfaces of the oil globules are stretched and pressed against each other and as this happens the emulsifier molecules will be dragged along the interface leaving regions with more and regions with fewer surfactant molecules at the O/W_2 interface (Leal-Calderon *et al.*, 2012). Moreover, if the adsorption of surfactant molecules to the O/W_2 interface to cover these gaps is slow the interfacial film becomes more susceptible to rupture (McClements, 2015). During swelling of $W_1/O/W_2$ globules the expansion of the O/W_2 interface results in decreased interfacial film strength and therefore making it more susceptible to rupture pressure (Geiger *et al.*, 1998; Geiger *et al.*, 1999). It may be that as the interface expands the distance between the Tween80 molecules at the interface increases and gaps will form. Moreover, excess Tween80 molecules in W_2 start to adsorb at the interface to fill up the free spaces caused by swelling and therefore maintain the O/W_2 interface and prevent the oil globule from bursting. The adsorption

of Tween80 at the interface occurs more rapidly when the excess Tween80 concentration in W_2 is high which prevents or slows down the rupture of the interfacial film. In contrast when the concentration of excess Tween80 in W_2 is low the adsorption of Tween80 at the interface is slower resulting in an expanding film that becomes progressively thinner and more susceptible to rupture. Also, an increase in continuous phase viscosity increases the time required for the interfacial films to drain to their critical thickness and therefore prolong its resistance to rupture (Shuster, 1985). The viscosity of the continuous phase was shown to increase when the amount of Tween80 was increased (Schmidts *et al.*, 2009). However, in the previous chapter (Table 5.1) it was shown that increasing the amount of Tween80 had no significant effect on viscosity of the continuous phase.

The release of *E. coli*-GFP was significantly higher at lower concentrations of PGPR. Geiger *et al.* (1999) found that by increasing the lipophilic surfactant concentration in the oil phase more rigidified O/ W_2 interface is formed measured as an increase in shear elastic modulus and interfacial tension. Furthermore, the authors suggested that an increase in lipophilic surfactant concentration increases the swelling capacity of the oil globule which delays breakdown and release of solutes. They argued that this occurred because of excess lipophilic surfactant in the oil phase diffusing to the O/ W_2 interface to fill up free spaces caused by the swelling (Geiger *et al.*, 1999). The viscosity of the oil phase will increase when more lipophilic surfactant is present in the oil phase (Leal-Calderon *et al.*, 2012). In the previous chapter (Table 5.1), it was shown that the viscosity of the oil phase significantly ($P < 0.05$) increased at higher amounts of PGPR. Jiao *et al.* showed that by increasing the concentration of Span80 (a lipophilic surfactant) the viscosity of the oil phase increased resulting in the ability of the oil globules to resist deformation upon applying force (by placing a coverslip

on top of the $W_1/O/W_2$ emulsion) (Jiao *et al.*, 2002; Jiao, and Burgess, 2003). Moreover, when present in excess in the oil phase, the lipophilic surfactant was found to increase the visco-elasticity of the interfacial film of the oil globule (Vasiljević *et al.*, 2006).

There was no significant difference in the release of *E. coli*-GFP between the different $W_1/O/W_2$ emulsions when the osmotic balance was unaltered after 45 and 90 minutes, however the release became higher with compared to without NaCl in W_2 after 180 minutes. NaCl can interact unfavourably with Tween80 and reduce the interfacial film strength of the $W_1/O/W_2$ globule (Jiao *et al.*, 2002) and this was shown to be concentration dependent (Opawale and Burgess, 1998). In comparison to sodium salicylate that was added to the W_2 phase of $W_1/O/W_2$ emulsions to alter the osmotic balance, NaCl led to a significant reduction in the strength of the interfacial film probably due to unfavourable interactions between NaCl and Tween80 and/or salting out (Jiao *et al.*, 2002).

In addition to oil globule bursting other mechanisms may have resulted in the release of *E. coli*-GFP. In the previous chapter, it was shown that coalescence between oil globules does not result in release of *E. coli*-GFP. The coalescence of W_1 droplets with the O/W_2 interface may result in release of *E. coli*-GFP but this mechanism was shown to occur more frequently with increasing concentrations of Tween80 (Ficheux *et al.*, 1998) which if was occurring in our study would have resulted in increased release of *E. coli*-GFP cells at high concentration of Tween80 but that would contrast with what is observed in this study. Further work is needed to find out if such mechanisms were responsible in the release of *E. coli*-GFP from the oil globules in hyper-osmotic solution.

It was found that the release of *E. coli*-GFP significantly ($P < 0.05$) increased at high amount of W_1 . The release of solutes from $W_1/O/W_2$ emulsion globules in hypo-osmotic solution increased with higher W_1 fraction (Cárdenas and Castro, 2003). When the W_1 volume is higher there was a significant increase in D (4, 3) and creaming thickness. Therefore, it may be that the interfacial film will be expanding at a faster rate when the W_1 concentration is high compared to when it is low and this will lead to more gaps to form at the O/W_2 interface which increase the chances of hole formation and propagation resulting in film rupture. The viscosity of the W_1/O emulsion increases with higher amounts of W_1 (Table 5.1). According to the Mooney equation (Mooney, 1951; Kita *et al.*, 1977) the increase in viscosity of $W_1/O/W_2$ emulsion globules at higher amounts of W_1 has a destabilising effect on the oil globules making them more susceptible to rupture (Jiao and Burgess, 2003). However, the increase in $W_1/O/W_2$ emulsion globule viscosity due to higher amount of W_1 phase has an opposite effect on emulsion stability than does the increase in the globule's viscosity due to higher amounts of lipophilic surfactant present in the oil phase (Jiao and Burgess, 2003).

When water migrates from W_2 to W_1 the volume of the continuous phase is reduced which can be observed as an increase in cream layer height. The stability of the cream layer was not affected by the presence of *E. coli*-GFP within the $W_1/O/W_2$ emulsion globules. Also, the presence of *E. coli*-GFP within the $W_1/O/W_2$ emulsion globules had no effect on the transport of water and/or the bursting of the oil globules. Moreover, the release mechanism had no effect on the viability of *E. coli* cells and there were no DiBAC₄ (3) stained *E. coli* cells. Since DiBAC₄ (3) is a membrane potential dye and will stain *E. coli* cells with a depolarised membrane

(Lewis *et al.*, 2004) our results indicate that no injury giving rise to depolarisation was caused to the *E. coli* cells after encapsulation and release from $W_1/O/W_2$ globules.

6.5 Conclusions

In this study, the release of *E. coli* cells from $W_1/O/W_2$ emulsion globules under hypo-osmotic pressure was investigated. The release of bacteria from the oil globules of $W_1/O/W_2$ emulsion was due to the bursting of the oil globules. Also bacterial viability was not affected by the release mechanism and structure of the $W_1/O/W_2$ emulsion. The release of *E. coli* cells can be modulated by changing the structure of the $W_1/O/W_2$ emulsion and was higher at low concentrations of Tween80 and PGPR and at high volumes of W_1 in the presence of NaCl in the W_1 phase. Also, it is important to note that the amount of encapsulated *E. coli*-GFP can vary from globule to globule and this, added to differences in globule sizes, results in some globules releasing more bacteria than others. However, in this study the overall behaviour of the $W_1/O/W_2$ emulsion can be obtained as an average of the bulk properties for these experiments. Moreover, to better understand the release mechanism it would be ideal to further investigate the bursting phenomenon in a more homogenous system in terms of globule size distribution and number of encapsulated bacteria. The possibility of modulating bacterial release from $W_1/O/W_2$ emulsion globules under hypo-osmotic pressure by changing its structure could be useful for industry to control and/or inhibit bacterial release along with the solutes contained within the W_1 phase.

CHAPTER 7: INCORPORATION OF $W_1/O/W_2$ EMULSION IN SET-TYPE YOGURT

7.1 Introduction

Yogurts are a suitable matrix for effective delivery of probiotics. The fortification of yogurts with probiotics can be achieved either by using probiotic as a starter culture or by introducing probiotics in the formulation during the fermentation process (Lourens-Hattingh and Vijoën, 2001). However, there are problems associated with the incorporation of probiotics in fermented dairy foods. The viability of probiotics during fermentation, storage and digestion of yogurt depends on a variety of factors including the production method, the bacterial species and strain, co-presence of other micro-organisms, culture conditions, final acidity, milk solid contents, sugar and salt concentration, dissolved oxygen, storage temperature, bile, and acid tolerance of some species (Farnworth, 2003). Also, probiotics can interfere with the starter cultures and therefore compartmentalisation is necessary.

The main factors that affect the survival of probiotics in yogurt are decrease in pH and accumulation of organic acids due to growth and fermentation (Hood and Zattola, 1988; Shah and Jelen, 1990). Prolonged storage at low temperature can also affect the viability of probiotics. Nighswonger *et al.* (1996) showed that the viability of three out of five strains of *L. acidophilus* and *L. casei* in yogurt was decreased due to refrigeration. Micro-encapsulation has been used as a solution for some of these problems. The encapsulation of *L. acidophilus* in alginate-starch beads protected the bacteria from gastrointestinal conditions and increased its viability (0.5-log) in yogurt after a 2-months storage period (Sultana *et al.*, 2000). $W_1/O/W_2$ can offer other benefits not possible with polymers including reduction of fat

(Lobato-Calleros *et al.*, 2008) and salt (Norton & Norton, 2010) and release of aroma and flavour (Malone *et al.*, 2003) while preserving taste perception. The encapsulation of *Lactobacillus* spp. probiotics in $W_1/O/W_2$ emulsions have shown to protect from cytotoxic gastric juice (Shima *et al.*, 2006; Pimentel-González *et al.*, 2009) bile salts, (Shima *et al.*, 2009), prolonged storage at low temperatures and during cheese manufacturing (Rodríguez-Huezo *et al.*, 2014) and melting (Xin *et al.*, 2009). However, studies on the ability of $W_1/O/W_2$ emulsion to protect probiotics during fermentation, storage and digestion of yogurt is lacking.

Many studies have investigated the incorporation of $W_1/O/W_2$ emulsion into dairy-based foods. Lobato-Calleros *et al.* (2006, 2008) studied the rheological and structural changes of low-fat white cheese (26% less oil content) made by substituting milk-fat with $W_1/O/W_2$ emulsion made with canola oil and stabilised by polymers and PGPR. They found no considerable difference in sensorial, textural, and rheological properties with $W_1/O/W_2$ emulsion substituted compared to milk-fat. In another study, low-fat Mozzarella cheese (13.2% less oil content) was produced by substituting milk-fat with $W_1/O/W_2$ emulsion made with corn oil and stabilised by various polymers and PGPR (Xu *et al.*, 2011). They found that $W_1/O/W_2$ emulsion improved greatly the stretchability and stickiness of the cheese by enhancing the role of cross-linking of proteins. Moreover, differences in microstructure was observed in across all the low-fat cheeses. Low-fat stirred yogurt (~19% less oil content) was produced using $W_1/O/W_2$ emulsion made with canola oil and stabilised by various polymers and PGPR (Lobato-Calleros *et al.*, 2009). Stirred yogurts containing $W_1/O/W_2$ emulsion showed higher stability compared to full milk-fat stirred yogurts depending on the type of polymer used to stabilise the $W_1/O/W_2$ emulsion. Lacunarity values (a measure of size distribution of gaps) and viscosity were higher in stirred

yogurts containing $W_1/O/W_2$ emulsions compared to full milk-fat stirred yogurts. During yogurt formation, the starter culture in milk ferments lactose to lactic acid leading to a decrease in pH (Tamime and Robinson, 2007). Milk caseins play an important role as thickening and gelling agents (McClements, 2005). As the pH drops, caseins form aggregates initiating the formation of a well-defined 3-D network. This network consists of protein clusters which are connected by thin strands leaving serum captivities (Tamime and Robinson, 2007). For set type yogurt, a firm gel with smooth homogeneous consistency, and no expelled whey at the surface are desired textural properties (Nöbel et al., 2016; Tamime and Robinson, 2007). Moreover, the presence of $W_1/O/W_2$ emulsion during acidification may alter the physicochemical characteristics and the stability of the yogurt structure.

The stability of emulsions within the food structure is also important to ensure a successful application. As mentioned previously, bacteria can alter the stability of emulsions and this mainly depends on the characteristics of the species such as metabolic activity, surface charge and hydrophobicity (Li *et al.*, 2001; Dorobantu *et al.*, 2004; Ly *et al.*, 2008; Boitard *et al.*, 2012; Firoozmand and Rousseau, 2014). On the other hand, emulsion structure can affect the bacteria by limiting the diffusion rate of nutrients and cause a reduction in the growth rates of bacteria (Brocklehurst *et al.*, 1995; Charteris, 1996).

The present study attempts to incorporate $W_1/O/W_2$ emulsion encapsulating the probiotic *L. paracasei* in a set-style yogurt during the fermentation process and investigates changes in textural and physicochemical properties as well as kinetics of bacterial growth. The stability of the yogurt containing the $W_1/O/W_2$ emulsion in terms of physicochemical and textural properties was also evaluated and the survival of *L. paracasei* was assessed after fermentation.

7.2 Materials and Methods

7.2.1 Materials

Includes all the materials in Chapter 4, Section 4.2.1, with the addition of the following:

- Pasteurised fresh whole milk (food grade) was purchased from a local retailer (United Kingdom).
- Butter (food grade) was purchased from a local retailer (United Kingdom).
- M17 agar (OXOID CM0785), fructose 98%, sodium hydroxide (NaOH) >98%, phenolphthalein (3,3-Bis(4-hydroxyphenyl)-1(3H)-isobenzofuranone), sodium sulphate (Na₂SO₄) >99%, Sudan red G >96%, lactic acid (2-Hydroxypropionic acid) >98%, skim milk powder (LP0031) and Whatman® membrane filters (13mm diameter; 0.45µm pore size) were purchased from Fisher Scientific (United Kingdom).

7.2.2 Microbial cultures

The microorganisms used in this study were *Lactobacillus paracasei* subsp. *paracasei* DC412 (Xanthopoulos *et al.*, 2000) generously provided by the Laboratory of Food Microbiology and Hygiene, Aristotle University of Thessaloniki and a commercially available freeze-dried yogurt starter culture (*Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* at ratio 1:1) was purchased from Micromilk® (Cremosano, Italy). *L. paracasei* was maintained on M.R.S petri dishes at 4°C. The freeze-dried yogurt starter culture was stored at 4°C.

7.2.3 Milk pre-treatment and inoculum preparation

To determine total soluble solids content of the milk 5 grams of milk was placed in a petri dish and left to dry in an oven at 100°C for 24 hours and then weighted. The following equation was used to calculate the total solid content of the milk:

$$\% \text{ total solids} = (\text{wt of petri dish} + \text{sample}) \text{ after drying} - (\text{wt of petri dish}) / (\text{wt of sample}) \times 100\% \quad (7.1)$$

The total soluble solid content of the milk was adjusted to 16% w/v with the addition of skimmed milk powder (SMP). After proper mixing, the fortified milk was transferred in sterile 500ml Duran bottles and pasteurized in a water bath at 80°C for 30 min. Inoculum was prepared by transferring aseptically 0.01 g of the freeze-dried yogurt starter culture in 150ml of sterile 10%w/v SMP solution. The culture was grown in an incubator at 37°C overnight without shaking resulting in an initial concentration of 2.3×10^9 CFU/ml for *S. thermophilus* and 1.8×10^9 CFU/ml for *L. bulgaricus*.

7.2.4 Butterfat oil isolation

Butterfat oil was isolated in accordance to the Council Regulation (EC) No 1255/1999 (Council regulation, 1999). The oil phase of the melted butter was purified by removing the water from the butter. Briefly, a butter bar was melted slowly in the oven at 50°C. A filter paper was folded into a cone shape and 10 grams of anhydrous sodium sulphate (Na_2SO_4) was added at the bottom of the cone shaped filter paper. The melted butter was transferred into the cone shaped filter paper and allowed to filtrate in an oven at 50°C. All the glassware was kept at the same temperature.

7.2.5 Preparation of $W_1/O/W_2$ emulsions

Double $W_1/O/W_2$ emulsions were prepared using a high shear mixer homogeniser (Silverson L5M) at room temperature using a two-step emulsification process (Figure A1). In the first step, primary W_1/O emulsions were made. An oil phase was prepared by dissolving 2 wt% PGPR in sunflower oil or butterfat oil. The inner aqueous phase (W_1) consisting of de-ionised water was emulsified (1700 rpm, 120 seconds) into the oil phase (W_1 : O phase ratio 40:60). In certain cases, the oil phase was stained using Sudan Red G (0.08% w/v) to monitor the mixing of the $W_1/O/W_2$ emulsion within yogurt. For microbial-encapsulation *L. paracasei* (10^8 CFU/ml) was harvested by centrifugation (10000 g, 10 minutes) from the growth medium and washed twice in PBS and re-suspended in fresh whole milk and used as the W_1 phase. In the second step $W_1/O/W_2$ emulsion was made. The previously prepared primary W_1/O emulsion was emulsified (2700 rpm, 60 seconds) into W_2 to form the $W_1/O/W_2$ emulsion (W_1/O : W_2 ratio of 20:80).

7.2.6 Preparation of yogurt

Yogurt fermentation was carried out in sterile Duran bottles (250ml) containing 150ml (or 100ml) of the pasteurized fortified milk. The substrates were inoculated with 6% (w/v) of the activated starter culture at room temperature. The acidification took place under static conditions in a water bath (Stuart) at 42°C and was monitored by pH measurements every 15 min as described by Lazaridou *et al.* (2014).

7.2.7 Incorporation of $W_1/O/W_2$ emulsion in yogurts

The $W_1/O/W_2$ emulsion made up 33% (v/v) of the yogurt. A 50ml of $W_1/O/W_2$ emulsion was introduced to the 100ml fermenting milk when the pH reached 5.7 ± 0.1 . The bottle was mixed gently by swirling and rotating three times and left to stand

until the end of the process when the pH reached 4.6 ± 0.05 . On completion of acidification, the bottles were cooled quickly by immersing them into ice water before storing them at 4°C for 24h (day 0). In every process, two bottles were used for the continuous pH monitoring of the control yogurt and the one with the $W_1/O/W_2$ emulsion.

7.2.8 Monitoring acidification kinetics

Physicochemical changes during fermentation were monitored by calculating the kinetics parameters in accordance to Mishra & Mishra (2013): V_{\max} (maximum acidification rate that measures the decrease of pH units per minute and the values are expressed as pH min), $t_{V_{\max}}$ (time to achieve the maximum acidification rate, in h), $pH_{V_{\max}}$ (pH at V_{\max}) and $t_{pH4.6}$ (time necessary to reach pH 4.6, in h). During acidification, a bottle was withdrawn every 1 h and analyzed for cell growth and selected physicochemical characteristics.

7.2.9 Bacterial enumeration

A representative sample of yogurt (approximately 1 g) was collected aseptically during sampling for bacteria enumeration. Cell counts were performed after serial dilutions in *PBS* and plating using the Miles & Misra technique (Miles *et al.*, 1938). Enumeration of *L. bulgaricus*, was done on modified M.R.S agar containing fructose whilst the *S. thermophilus* and *L. paracasei* was done on M17. Only *L. bulgaricus* will grow on modified M.R.S agar containing fructose (Tabasco *et al.*, 2007). On M17 agar, only *S. thermophilus* will grow at 35°C while *L. paracasei* and *S. thermophiles* will grow at 45°C (Kristo *et al.*, 2003). The plates were incubated at 35°C or 45°C for 48 h and the number of *L. paracasei* was calculated by subtracting the log CFU/g of M17 agar at 45°C from 35°C.

7.2.10 Observation of $W_1/O/W_2$ emulsions within yogurt

7.2.10.1 Optical and fluorescence microscopy

This procedure was done as in Section 4.2.4.

7.2.10.2 Scanning electron microscopy

The microstructure of $W_1/O/W_2$ emulsion and yogurt with the double emulsion was visualised using a Cryogenic scanning electron microscopy (Cryo-SEM; Philips XL30 FEG ESSEM). One drop of the sample was frozen to -180°C in liquid nitrogen slush. Fracturation and etching of the frozen sample was performed for 5 min at -195°C inside a preparation chamber. Subsequently, samples were sputter coated with gold and scanned at -160°C . Periodic addition of liquid nitrogen to the system maintained the low temperature during observation.

7.2.11 Measurement of W_1 droplets and oil globule size of $W_1/O/W_2$ emulsions

The particle size distribution of W_1 droplets in primary O/W_1 and oil globules in the $W_1/O/W_2$ emulsion was measured immediately after preparation and as a function of storage time using a laser diffraction particle size analyser (Malvern Mastersizer 2000, Malvern Instrument Ltd, Worcestershire, UK), equipped with a He-Ne laser ($\lambda = 633 \text{ nm}$). This procedure was done as in Section 3.2.6.1.

7.2.12 Analytical Determinations

7.2.12.1 Total acidity

Total acidity was determined by titration with 0.1 N NaOH. A small sample of yogurt (9 g) was dissolved in 18ml of water. The diluted samples were titrated using 0.1 N

NaOH using phenolphthalein solutions (1% w/v) as an indicator. The total acidity was expressed as % w/w lactic acid and calculated using the formula 1 ml 0.1 NaOH = 0.009 g lactic acid.

7.2.12.2 Water retention capacity

Water retention capacity was determined by centrifugation. Approximately 10 g of the sample was transferred in a pre-weighted conical tube and were centrifuged at (10,000 g, 10 minutes) for 10 minutes. The supernatant was dispensed and water retention capacity was calculated as % (w/w) of the sediment over the initial weight of the sample.

7.2.12.3 Syneresis

Syneresis is the extraction of whey from the yogurt gel that determines its quality and stability. Approximately 5 g of the sample was weighted and placed over filter paper (Whatman Grade 1) and drained under vacuum for 10 minutes. The drained liquid was weighed and syneresis was expressed as the %w/w of the drained liquid over the initial weight of the sample.

7.2.12.4 Viscosity measurements

Rheological characterisation of emulsion separate components was done by measuring the viscosity of the yogurt samples with and without $W_1/O/W_2$ emulsions. The measurement was performed at 25°C using AR-G2 rheometer (TA instruments, New Castle, Delaware USA) equipped with a vane spindle (diameter 14mm). The apparent viscosity of a representative yogurt sample (~30ml) was measured over a shear rate 700-1000 s⁻¹.

7.2.12.5 Texture analysis

Texture of the yogurt is a major quality component. For texture analysis, a sample of yogurt (30ml) was transferred to a cylindrical plastic vessel (diameter 140mm) immediately after preparation and left to set for another 24h at 4°C. Texture profile analysis (TPA) of the samples was conducted using a Texture Analyzer TAXT2i (Stable Micro Systems, Surrey, England) accompanied with a computer software (Exponent). Samples were compressed under a cylindrical probe (P/40) at a test speed of 1 mm/s and a trigger force of 1 g, using the Texture Analyzer. Two compression cycles at 50% of the initial height were applied using a post-test speed of 4 mm/s. The data obtained from the force–time curves were used to calculate the following parameters:

- hardness (g) - force necessary to attain a given deformation,
- cohesiveness - strength of internal bonds making up the body of the product,
- adhesiveness (g*s) - work required to pull food away from a surface,
- gumminess (g) - energy required to disintegrate a semi-solid food product to a state ready for swallowing.

7.2.13 Statistical analysis

Statistical analysis was done as described in Section 3.2.10 and each experiment was conducted in triplicate (N=3).

7.3 Results

7.3.1 Characterisation of primary W_1/O and encapsulation of *L. paracasei*

In this study, it was ensured that the homogenisation conditions were such that the average mean size distribution [D (4, 3)] of W_1 droplets (10-15 μ m) (Appendix, Figures A13 and A14) and $W_1/O/W_2$ emulsion globules (50-60 μ m) (Appendix, Figures A15 and A16) were suitable for encapsulating *L. paracasei* probiotic. *L. paracasei* cells were successfully entrapped within the $W_1/O/W_2$ emulsion (Appendix, Figure A17).

7.3.2 Incorporation of $W_1/O/W_2$ emulsion into yogurt

Two $W_1/O/W_2$ emulsion systems made with different dispersed oil phase (butterfat or sunflower oil) were tested for their effect on yogurt formation and its stability during storage. Butterfat was used due to it being natural to the yogurt formulation while sunflower oil was used as a substitute. Vegetable oils have been used as a replacement to milk fat in set type yogurts as a healthier substitute (Farmani *et al.*, 2016). The microstructure of yogurt with $W_1/O/W_2$ emulsion was observed using optical microscopy. Immediately after the acidification process the $W_1/O/W_2$ emulsion globules made with butterfat were dispersed homogeneously throughout the yogurt but flocculation and partial coalescence between the oil globules was observed (Figure 7.1A and B). The $W_1/O/W_2$ emulsion made with sunflower oil was dispersed homogeneously throughout the yogurt and no flocculation or coalescence was observed (Figure 7.2C and D) at the end of the fermentation process. Based on these results sunflower oil was used as the dispersed phase in further experiments.

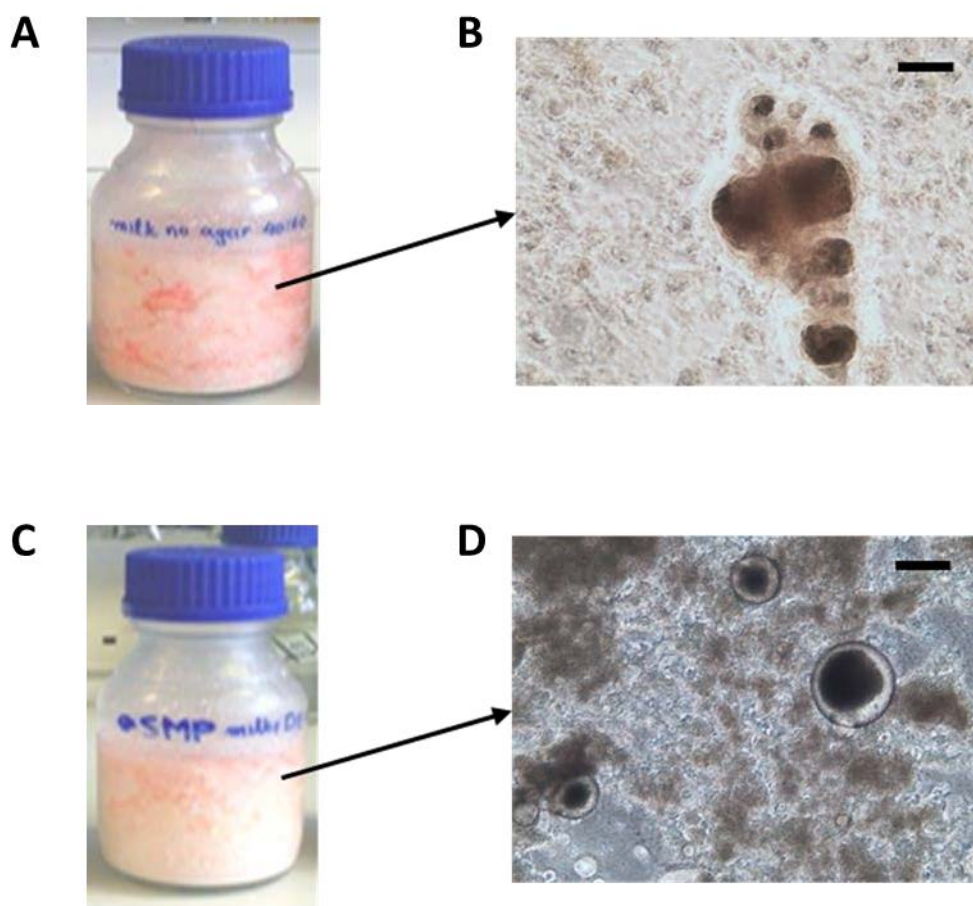


Figure 7.1 Photographic (right) and microscopic (left) images of the yogurt at the end of the acidification process containing $W_1/O/W_2$ emulsion made with butterfat (A and B) or sunflower oil (C and D) as the dispersed oil phase. The $W_1/O/W_2$ emulsion was stained with Sudan red. Scale bar: 100 μ m.

7.3.3 Effect of $W_1/O/W_2$ emulsion on yogurt structure and characteristics

Physicochemical properties of the milk changed rapidly during the acidification process. The drop of pH (Figure 7.2) was accompanied with a gradual increase in acidity content (Figure 7.3). The accumulation profile of lactic acid was similar in all the yogurt samples having a significantly similar content of lactic acid ($\sim 0.75\%$ w/w) at the end of the acidification process.

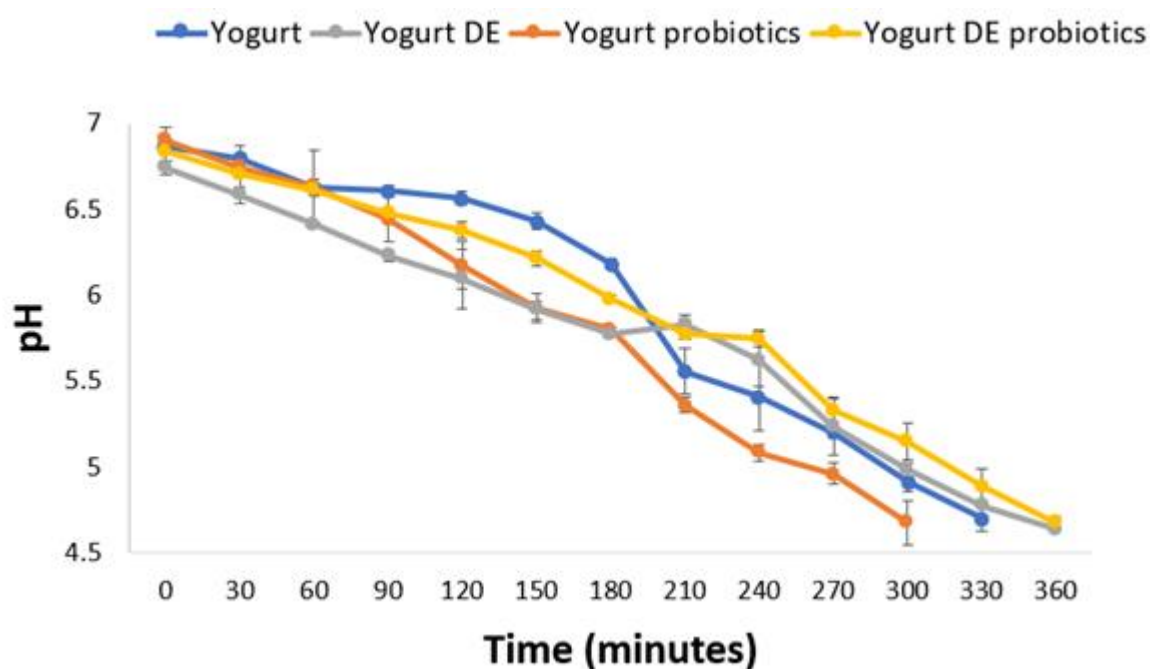


Figure 7.2 Acidification profile (pH values) of fermented milk with and without the addition of $W_1/O/W_2$ emulsion (DE) and/or *L. paracasei* probiotic. Bars represent mean \pm standard deviation taken from a minimum of 3 independent experiments.

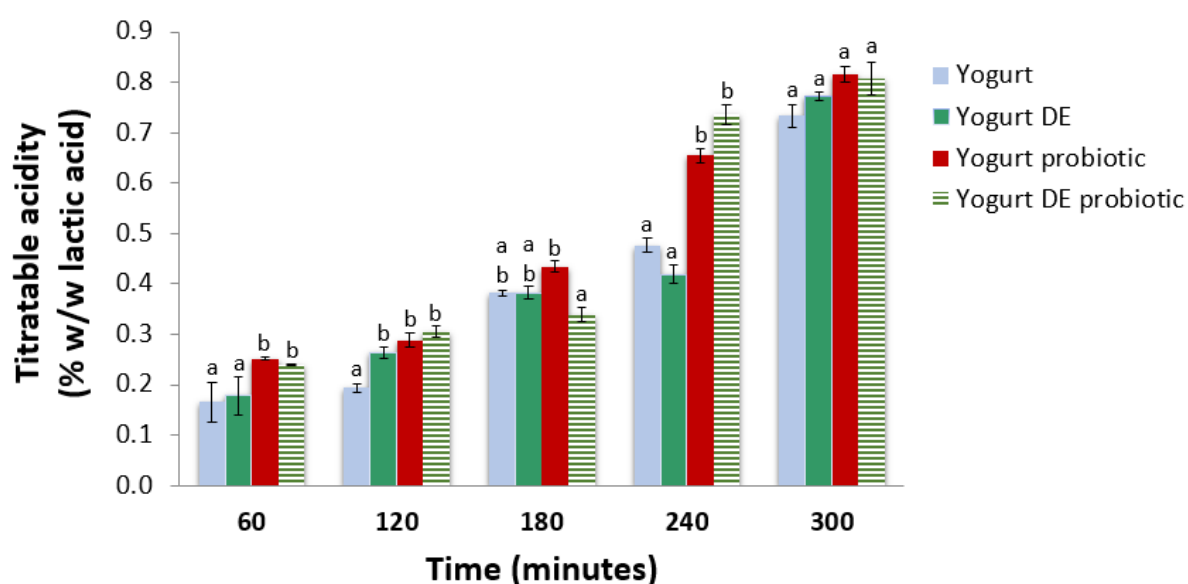


Figure 7.3 Titratable acidity results during acidification process of milk with and without the addition of $W_1/O/W_2$ emulsion (DE) and/or *L. paracasei* (probiotic). Bars represent mean \pm SEM taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different (P < 0.05). The data was analysed with one-way ANOVA.

The acidification profiles and V_{\max} , $T_{V_{\max}}$, $pH_{V_{\max}}$, $t_{pH4.6}$ were calculated for the yogurt with and without $W_1/O/W_2$ emulsion (Table 7.1). Understanding acidification kinetics is essential to yogurt production as prolonged acidification can lead to undesirable physicochemical and sensorial properties (Mishra and Mishra, 2013). Some variation was observed between the two processes. The maximum acidification rate, V_{\max} , was significantly ($P<0.05$) higher (~1.4-1.6 times) without compared to with $W_1/O/W_2$ emulsion and/or probiotic. The V_{\max} was reached earlier without compared to with $W_1/O/W_2$ emulsion (210 min vs 270 min). Interestingly, $pH_{V_{\max}}$, was significantly ($P<0.05$) higher without compared to with probiotic. Furthermore, the overall duration of the process, $T_{pH4.6}$ (min), was reached earlier without compared to with $W_1/O/W_2$ emulsion. These results suggest that the addition of the $W_1/O/W_2$ emulsion reduced the rate of acidification.

Table 7.1 Acidification parameters (V_{\max} , $T_{V_{\max}}$, $pH_{V_{\max}}$, $t_{pH4.6}$) of fermented milk with and without $W_1/O/W_2$ emulsion and/or *L. paracasei* probiotic. Data taken from a minimum of 3 independent experiments.

	V_{\max} (10^{-3} pH units/min)	$T_{V_{\max}}$ (min)	$pH_{V_{\max}}$	$T_{pH4.6}$ (min)
Yogurt	20.91 ± 3.52^a	210	5.56 ± 0.13^a	330
Yogurt DE	13.02 ± 1.20^b	270	5.24 ± 0.06^{ab}	360
Yogurt probiotic	14.70 ± 1.51^b	210	5.36 ± 0.06^b	300
Yogurt DE probiotic	14.04 ± 0.90^b	270	5.33 ± 0.07^b	360

The data was analysed with one-way ANOVA. ^{a,b,c} Mean \pm standard deviation with different letters are significantly different ($P<0.05$).

Syneresis (i.e. the whey separation) decreased during the acidification process (Figure 7.4) All samples had significantly similar syneresis percentage at the end of the acidification process. In all samples, water retention capacity was increased during the acidification process as the yogurt structure started forming (Figure 7.5). Overall, a sharp increase in the ability of all the samples to retain water was

recorded after 180 minutes of fermentation with the pH values being approximately ~5.7. However, samples containing $W_1/O/W_2$ emulsion exhibited significantly ($P<0.05$) lower increase rates and final values of water retention capacity.

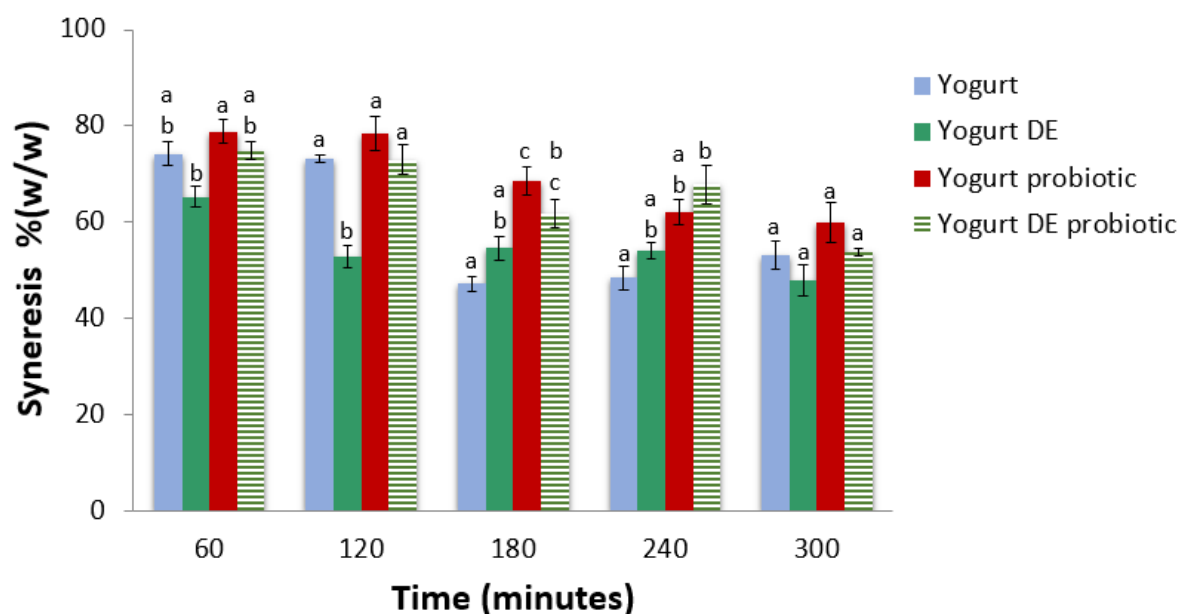


Figure 7.4 Syneresis results during acidification process of milk with and without the addition of $W_1/O/W_2$ emulsion (DE) and/or *L. paracasei* (probiotic). Bars represent mean \pm SEM taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different ($P<0.05$). The data was analysed with one-way ANOVA.

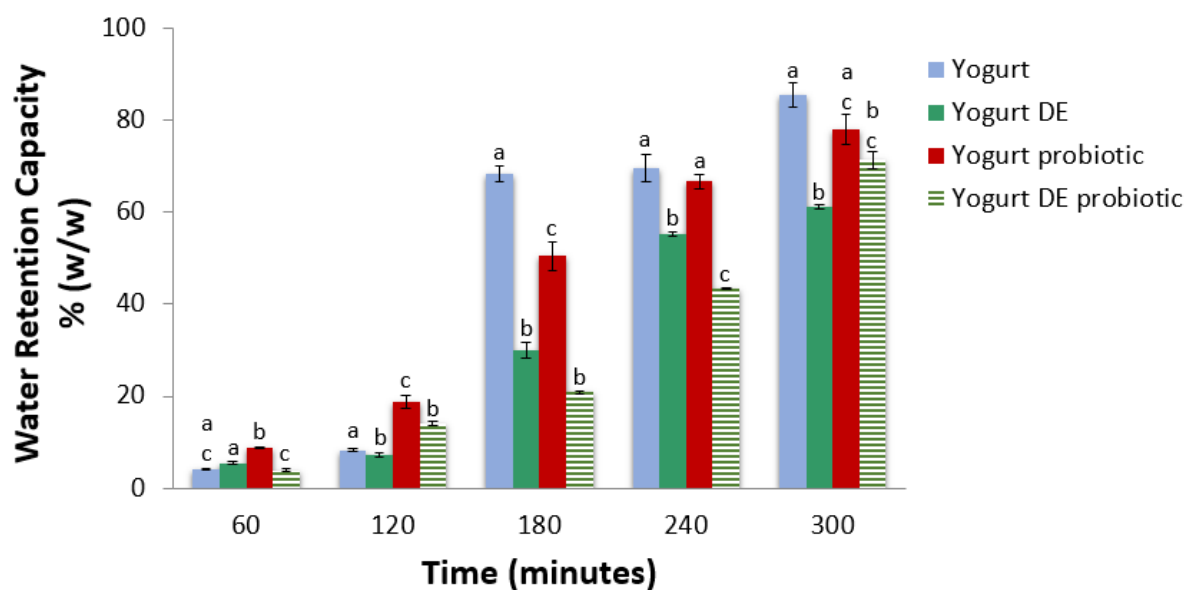


Figure 7.5 Water retention capacity during acidification process of milk with and without the addition of $W_1/O/W_2$ emulsion (DE) and/or *L. paracasei* (probiotic). Bars represent mean \pm SEM taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different ($P < 0.05$). The data was analysed with one-way ANOVA.

The viscosity of the pasteurised milk containing 16% w/v total soluble solids in the beginning of the fermentation had a value of 9.98 mPa.s. During the initial stages of the acidification process, when the pH was still high, a slight increase in viscosity was observed (Figure 7.6 and Table A5). After 180 minutes of acidification, the viscosity values of all samples were almost doubled marking the onset of the formation of this acid induced gel. The introduction of the $W_1/O/W_2$ emulsion to the fermenting system led to a small decrease in the viscosity after 240 minutes. At 240 minutes and up to the end of the acidification process, the viscosity was significantly ($P < 0.05$) higher in samples without compared to with $W_1/O/W_2$ emulsion.

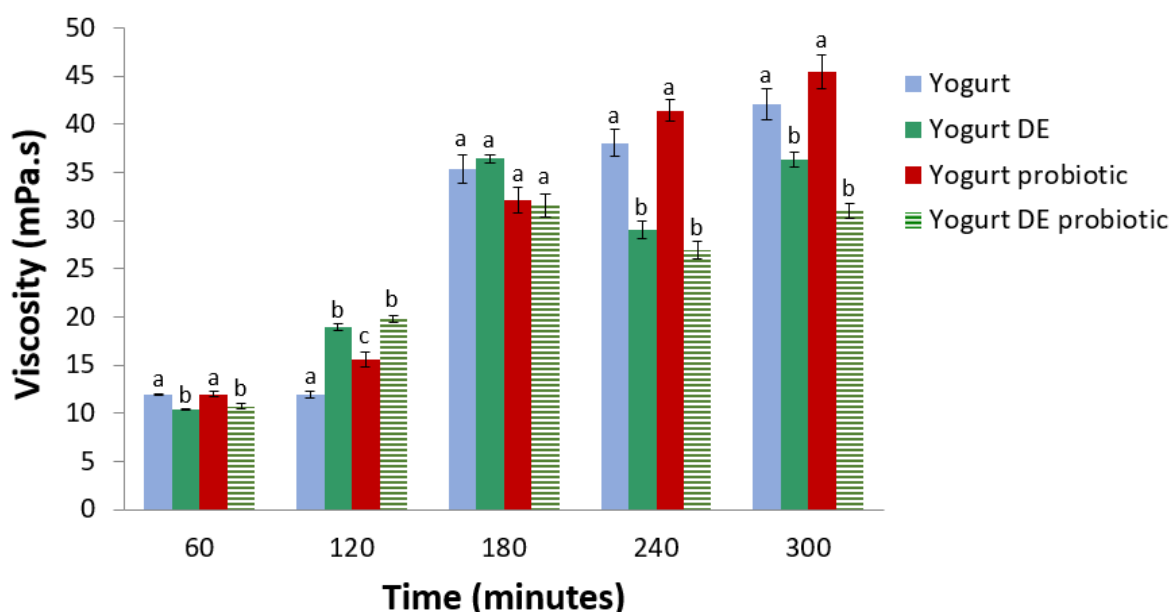


Figure 7.6 Viscosity results during acidification process of milk with and without the addition of $W_1/O/W_2$ emulsion (DE) and/or *L. paracasei* (probiotic). The apparent viscosity was measured over a shear rate range $700-1000\text{ s}^{-1}$ and taken as the average of the final 7 points where the curve becomes a plateau. Bars represent mean \pm SEM taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different ($P < 0.05$).

7.3.4 Effect of $W_1/O/W_2$ emulsion on bacterial population

The population evolution of *L. bulgaricus*, *S. thermophilus* and *L. paracasei* during the acidification process is shown in Figures 7.7, 7.8 and 7.9. The *S. thermophilus* population in all the samples fluctuated during the acidification process and eventually they all were within the range of 8.1 to 8.9-log CFU/g (Figure 7.7). The *S. thermophilus* population in all samples containing $W_1/O/W_2$ emulsions follow a similar trend. The sample containing no probiotic and/or $W_1/O/W_2$ emulsion showed a steady increase in population up to 180 minutes followed by a decrease until the end of the acidification process. The sample containing un-encapsulated probiotic showed a slower increase in population reaching its maximum population at 240 minutes. The *L. bulgaricus* population in all the samples remained relatively constant until the end of the acidification process (8 to 9-log CFU/g) (Figure 7.8).

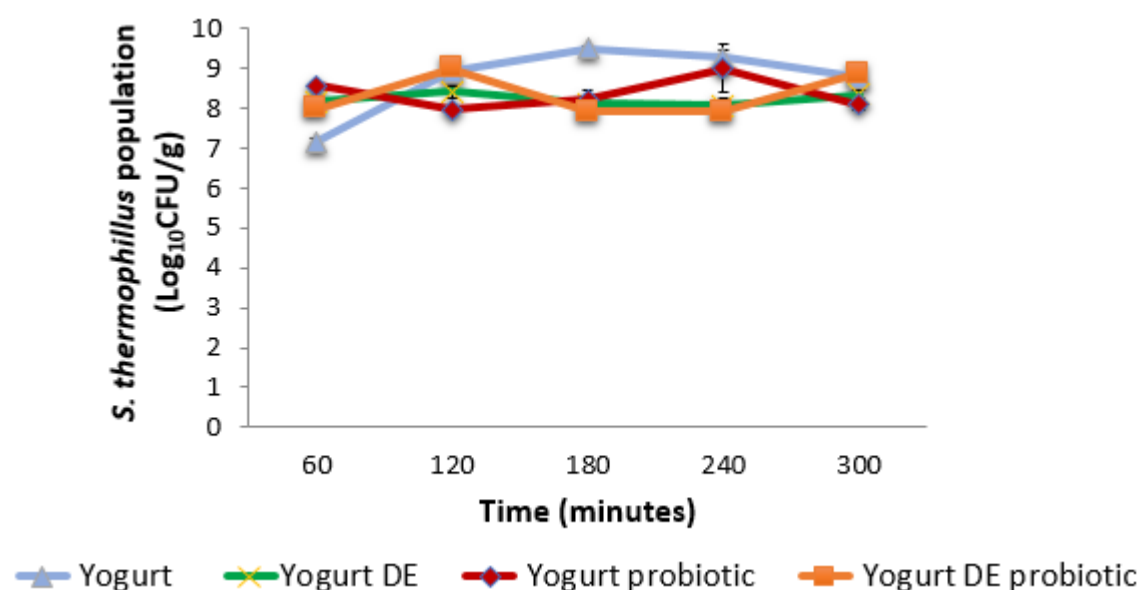


Figure 7.7 Cell viability of *S. thermophilus* with and without the addition of W₁/O/W₂ emulsion (DE) and/or *L. paracasei* (probiotic) during the acidification process of milk. Bars represent mean \pm SEM taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different ($P < 0.05$). The data was analysed with one-way ANOVA.

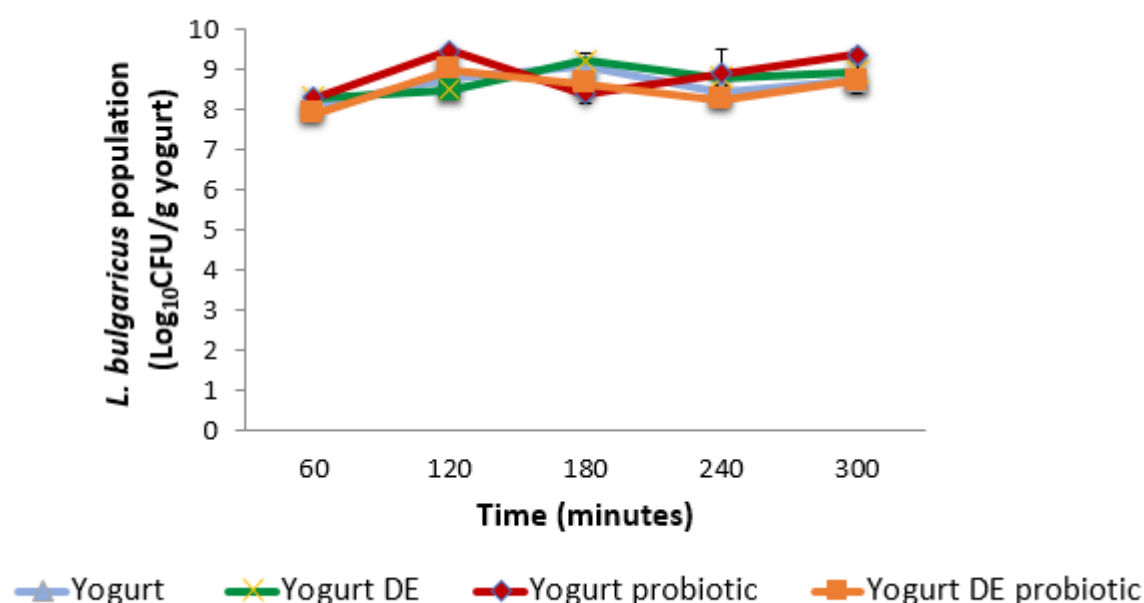


Figure 7.8 Cell viability of *L. bulgaricus* with and without the addition of W₁/O/W₂ emulsion (DE) and/or *L. paracasei* (probiotic) during the acidification process of milk. Bars represent mean \pm SEM taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different ($P < 0.05$). The data was analysed with one-way ANOVA.

There was no significant difference in *L. paracasei* population at 180 minutes, however, the *L. paracasei* population was significantly ($P<0.05$) higher without compared to with $W_1/O/W_2$ emulsion at 240 and 300 minutes (Figure 7.9). These results suggest that the presence of $W_1/O/W_2$ emulsion does not affect the growth kinetics of *L. bulgaricus* and *S. thermophilus* while the encapsulation of *L. paracasei* limited its ability to grow.

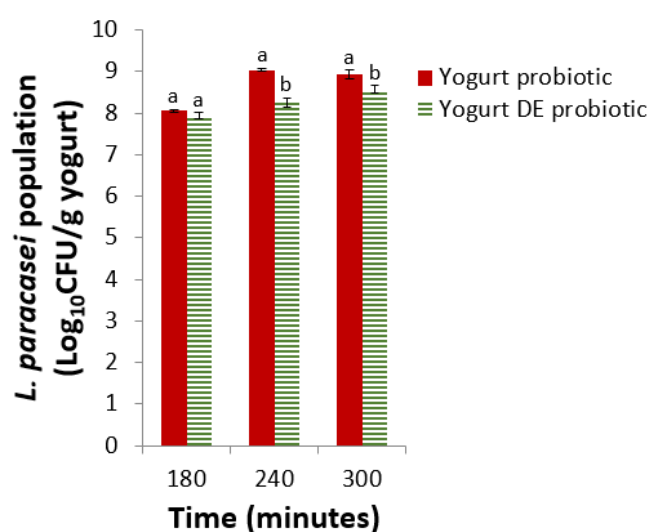


Figure 7.9 Cell viability of *L. paracasei* with and without the addition of $W_1/O/W_2$ emulsion (DE) and/or *L. paracasei* (probiotic) during the acidification process of milk. Bars represent mean \pm SEM taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different ($P<0.05$). The data was analysed with one-way ANOVA.

7.3.5 Effect of $W_1/O/W_2$ emulsion on the stability of yogurt

7.3.5.1 Textural analysis

The textural characteristics of the yogurt with or without $W_1/O/W_2$ emulsion and/or probiotics was investigated in terms of hardness, cohesiveness, gumminess, and adhesiveness (Table 7.2). Hardness was significantly ($P<0.05$) higher in yogurt

without compared to with $W_1/O/W_2$ emulsion and/or probiotics. Yogurts containing probiotics exhibited significantly ($P<0.05$) higher values of cohesiveness. Gumminess (i.e. required more energy to disintegrate) was significantly ($P<0.05$) higher in yogurt without compared to with $W_1/O/W_2$. The adhesiveness values were significantly ($P<0.05$) lower in yogurts with compared to without probiotic and/or $W_1/O/W_2$ emulsion. These results suggest that the presence of $W_1/O/W_2$ emulsion and/or probiotics in the yogurt structure altered the values of major textural parameters.

Table 7.2 Effect of $W_1/O/W_2$ emulsion on the texture profile of yogurts after fermentation. Data taken from a minimum of 3 independent experiments.

	Hardness	Cohesiveness	Gumminess	Adhesiveness
Yogurt	79.23±12.91 ^a	0.82±0.04 ^a	64.67±10.03 ^{abc}	-150.52±16.09 ^a
Yogurt DE	67.43±7.73 ^a	0.72±0.04 ^b	48.54±6.23 ^{bc}	-88.70±7.56 ^b
Yogurt probiotic	65.13±11.01 ^a	1.24±0.07 ^c	61.14±6.92 ^a	-91.68±5.19 ^b
Yogurt DE probiotic	70.83±5.51 ^a	0.75±0.06 ^{ab}	53.25±5.03 ^c	-97.48±8.38 ^b

The data was analysed with one-way ANOVA.

^{a,b,c} Mean ± standard deviation with different letters are significantly different ($P<0.05$).

7.3.5.2 Microscopic observation

To study the stability of the $W_1/O/W_2$ emulsion, the structure of the yogurts containing $W_1/O/W_2$ emulsion was observed using cryo-SEM, optical and fluorescence microscopy Figure 7.10, 7.11, 7.12 and 7.13. Images of cryo-fractured yogurt samples show successful incorporation of $W_1/O/W_2$ emulsion as the oil globules seem to be part of the gel network of the yogurt (Fig 7.10). The inner W_1 phase of the oil globules was observed within the $W_1/O/W_2$ emulsion at the end of the acidification process (Fig 7.11).

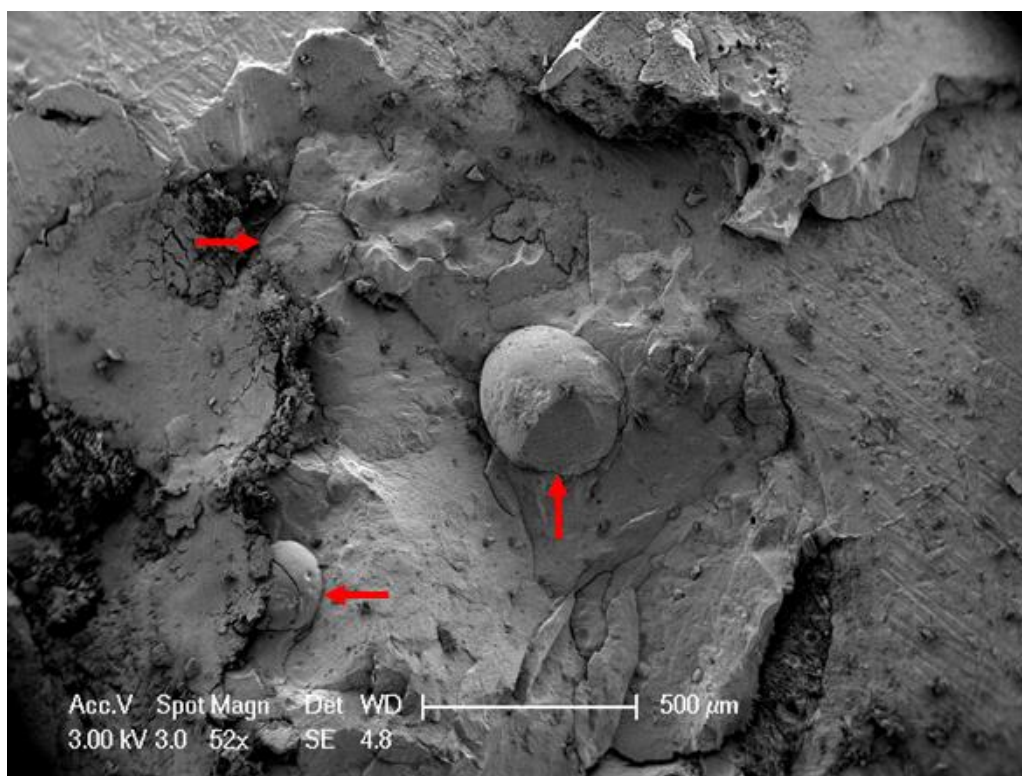


Figure 7.10 Cryo-SEM images of yogurt containing $W_1/O/W_2$ emulsion immediately after the acidification process. The oil globules in the gel-network of the yogurt indicated by the red arrows.

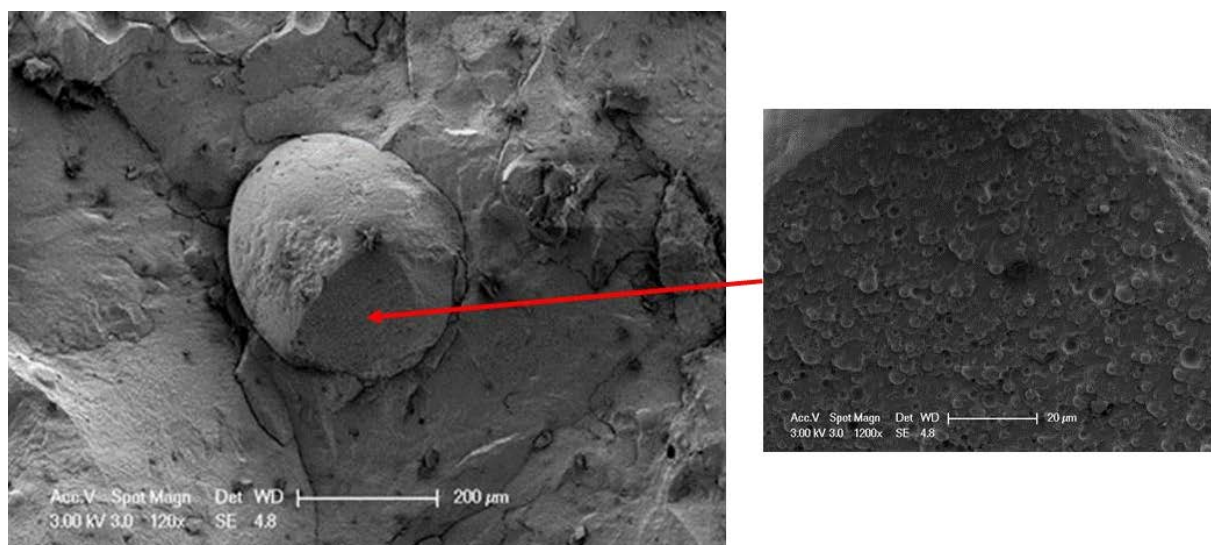


Figure 7.11 Cryo-SEM images of the W_1 droplets within the $W_1/O/W_2$ emulsion globules immediately after the acidification process.

Although some inner W_1 phase was partially lost in some oil globules, most the oil globules retained their inner W_1 phase at the end of acidification process (Figure 7.12). Furthermore, no flocculation or aggregation between the globules was observed (light microscopy). Fluorescence microscopy images show the presence of the *L. paracasei* probiotic within the oil globules and the starter culture within the gel network of the yogurt (Figure 7.13).

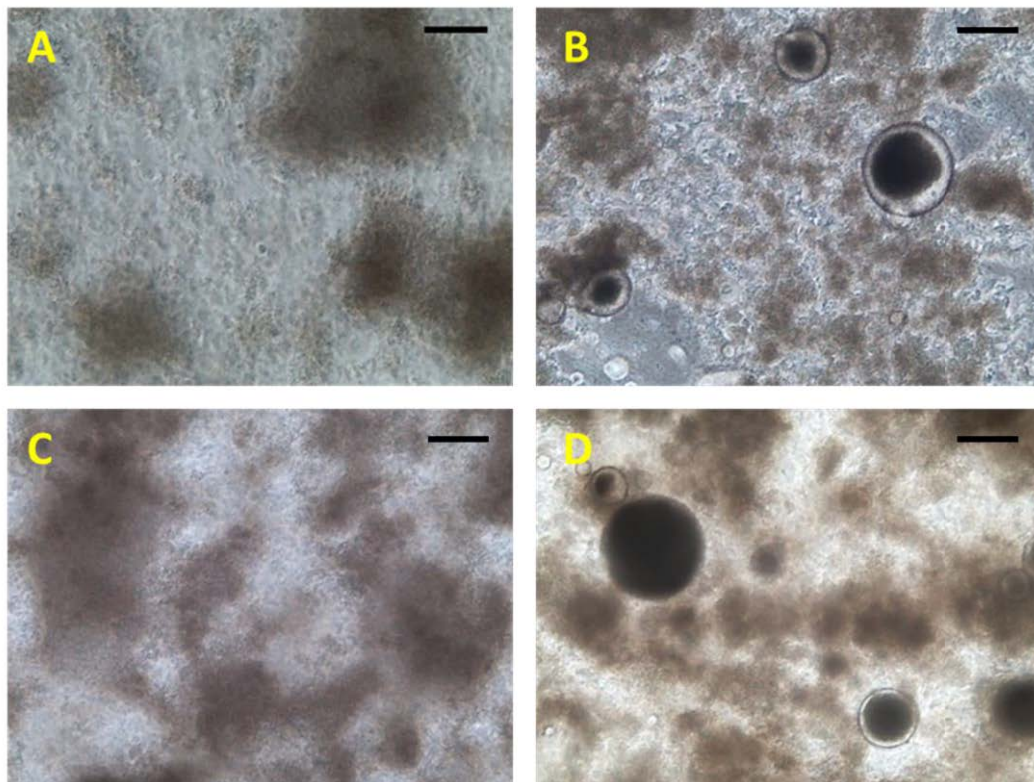


Figure 7.12 Optical images of the yogurt samples with or without $W_1/O/W_2$ emulsion globules and/or probiotics immediately after the acidification process. The yogurts samples were as follows: without $W_1/O/W_2$ emulsion or probiotics (A), with $W_1/O/W_2$ emulsion but no *L. paracasei* probiotic (B), without $W_1/O/W_2$ emulsion but contained *L. paracasei* probiotics, (C), with $W_1/O/W_2$ emulsion encapsulating *L. paracasei* probiotic (D). Scale bar: 100 μ m.

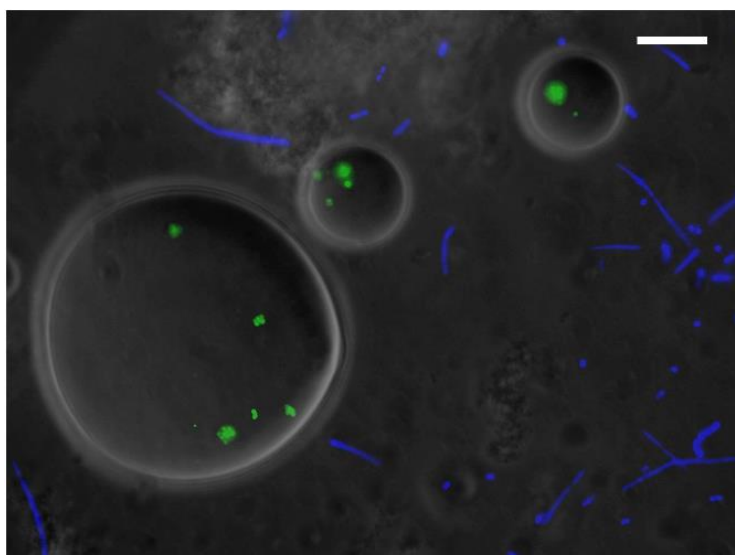


Figure 7.13 Fluorescence image of the yogurt samples with $W_1/O/W_2$ emulsion immediately after the acidification process showing the starter culture (blue) within the W_2 phase and the *L. paracasei* probiotic (green) within the oil globules. Scale bar: 10 μ m.

7.4 Discussion

In the first set of preliminary experiments, butterfat was used as the dispersed phase since it is compatible with the yogurt system. Butterfat is partially solid at room temperature forming a three-phase system that consists of water, oil and crystals (van Boekel and Walstra, 1981). These crystals can pierce the interfacial film of the $W_1/O/W_2$ emulsions causing the oil globules to coalesce (Frasch-Melnik *et al.*, 2010a; Xu *et al.*, 2005). Based on microscopic observation the butterfat emulsion was not suitable for the experiment and thus it was discontinued. The $W_1/O/W_2$ emulsion was not stable as the oil globules were coalesced and flocculation was observed at the end of the acidification process. Such instabilities were not observed with sunflower oil and therefore it was the preferred ingredient to be used as the dispersed phase for this study. Using cryo-SEM it was shown that the oil globules of the $W_1/O/W_2$ emulsion maintained the inner W_1 phase at the end of the acidification process. Moreover, no aggregation or flocculation was observed between the oil globules which seem to be part of the gel network of the yogurt.

The difference detected in the acidification profile was also reflected in the corresponding acidification parameters. The V_{\max} values recorded in the yogurt with the $W_1/O/W_2$ emulsion are comparable with the range of those reported for yogurts fermented with other starters from cow milk ($19.89\text{--}23.44 \times 10^{-3}$ pHunits/min) (Medeiros *et al.*, 2014). Slightly lower V_{\max} values ($13.96\text{--}15.66 \times 10^{-3}$ pHunits/min) have been recorded in literature with the addition of other ingredients in the system (e.g. passion fruit peel powder) since the latter can contain compounds that interfere with the buffering capacity of the milk (do Espírito Santo *et al.*, 2012). In this study, the V_{\max} values of yogurt without probiotic and/or $W_1/O/W_2$ emulsion was significantly ($P < 0.05$) higher (~ 1.5 -fold) compared to all the other samples. Also, $pH_{V_{\max}}$ values of yogurt without probiotic and/or $W_1/O/W_2$ emulsion was higher compared to all the other samples. These results can be attributed to the fact that during the yogurt production the system is left to stand during the whole process whereas in the case of the yogurts with probiotic and/or $W_1/O/W_2$ emulsion, the ingredients were introduced after 180 minutes by gently mixing which is likely to cause slight disturbances in the system and in the acidification process.

As expected, in all samples tested the drop of pH was accompanied by a gradual increase in the acidity content. The recorded values are comparable to literature data for yogurts obtained by cow milk fermentation (do Espírito Santo *et al.*, 2012). In this study $T_{V_{\max}}$ was achieved later during the acidification process with compared to without $W_1/O/W_2$ emulsion (210 min vs 270 min). Similarly, $T_{pH4.6}$ was prolonged in samples with compared to without $W_1/O/W_2$ emulsion. When $W_1/O/W_2$ emulsion is added during the acidification process, the constituents of the unfermented milk (e.g. soluble phosphate, colloidal calcium phosphate, casein, and whey proteins) of the outer phase affect its buffering capacity (Salaün *et al.*, 2005). Thus, when a portion

of the W_2 phase (unfermented milk containing the W_1/O emulsion) is introduced to the fermenting system it can alter its acidification kinetics.

The ability of the yogurt to retain water was affected by adding the $W_1/O/W_2$ emulsion. The water retention capacity was significantly ($P<0.05$) higher in samples without compared to with $W_1/O/W_2$ emulsion at 180, 240 and 300 minutes. Most likely the presence of the oil globules partially disrupted the gel formation, altering the ability of the structure to retain water. There was no significant difference in syneresis values between all samples at the end of the acidification process. Syneresis marks the deterioration of the protein network and the subsequent loss of the serum phase from the yogurt gel (Lucey, 2002). Despite the fact that the stirring stage during the introduction of the $W_1/O/W_2$ emulsion to the yogurt was expected to stimulate syneresis (Ozturkoglu-Budak *et al.*, 2016), however, this was not the case in the present study. In literature, syneresis values seem to be inversely related to fat content, i.e. increased fat content reduces the whey released (Akgun *et al.*, 2016; Isanga and Zhang, 2009). Furthermore, in this study the amount of oil added to the system after incorporating $W_1/O/W_2$ emulsion during the acidification process is probably too low (~6.6%) to significant cause changes in syneresis.

Changes in viscosity of the samples without $W_1/O/W_2$ emulsion verified the three-step structure formation process proposed by Parnell-Clunies *et al.* (1998), i.e. an initial lag period of low viscosity followed by a period of rapid increase and a final stage of high viscosity. The introduction of the $W_1/O/W_2$ emulsion to the fermenting system led to a decrease in the apparent viscosity after 240 minutes, indicating a disturbance in the yogurt structure formation. A well-defined 3-D network is formed as the pH drops initiated by caseins that form aggregates at $pH<5.2$ (Tamime and Robinson, 2007). The strength and number of bonds between the micelles as well as

their structure and spatial distribution affect the viscosity of the yogurt (Lucey *et al.*, 1998). Casein micelles start to aggregate at a pH close to ~5.3, which also causes the solubilisation of colloidal calcium phosphate and the change in viscosity (Mishra and Mishra, 2013). Overall, the presence of $W_1/O/W_2$ emulsion in the yogurt decreased significantly ($P<0.05$) the viscosity compared to yogurt without $W_1/O/W_2$ emulsion. Similar observations were made by Izadi *et al.* (2010) with the addition of W/O emulsion to yogurt. In this study, the $W_1/O/W_2$ emulsion was mixed with the fermenting milk at pH values close to ~5.7. At such pH values the structure that is forming is still weak and was probably partially disrupted by the presence of the oil globules resulting in a yogurt with a significantly ($P<0.05$) lower viscosity value compared to the sample without $W_1/O/W_2$ emulsion immediately after the acidification process.

The introduction of $W_1/O/W_2$ emulsion did not affect the growth kinetics of *S. thermophilus* as all the samples had similar counts at the end of the acidification process. Also, depending on the sample, the maximum population of *L. bulgaricus* (~9 log₁₀CFU/g yogurt) was reached after 120 to 180 minutes and remained rather constant throughout the acidification process. *L. bulgaricus* is stimulated by the formate produced by *S. thermophilus* in the symbiotic fermentation (Zourari *et al.*, 1992). The similar growth pattern of *L. bulgaricus* exhibited between all the samples indicate that the symbiotic phenomena occurring during acidification was not interrupted when the $W_1/O/W_2$ emulsion was introduced. The *L. paracasei* probiotic population was significantly ($P<0.05$) higher when un-encapsulated compared to encapsulated at 240 and 300 minutes of the acidification process. Since *L. paracsei* probiotic grows optimally at pH 4.5-5.7 (Mahboubi and Kazempour, 2016), the un-encapsulated probiotic would be expected to grow when added during acidification

(pH <5.7) while the encapsulated probiotic will be present in high pH values (pH 6-7) that would not encourage its growth. Also, microscopic observation showed that the *L. paracasei* probiotic remained within the oil globules which is important to protect from the harsh environment of the stomach. Encapsulation within $W_1/O/W_2$ emulsion protects the probiotic bacteria from the acidic conditions of the stomach and bile salts.

Textural and rheological properties of yogurts are determined to a great extent by their internal structure (Yang and Li, 2010) and they provide an insight about the sensory characteristics of the product (Szczesniak, 2002). Set-type yogurts should be firm but spoonable (Tamime and Robinson, 2007), thus hardness, cohesiveness, adhesiveness and gumminess are considered important for this type of yogurts (Domagala *et al.*, 2006). Hardness is among the most commonly evaluated characteristic and expresses the force necessary to attain a given deformation (Uprit and Mishra, 2004). The lower hardness values observed for yogurts containing the $W_1/O/W_2$ emulsion and/or probiotics can be attributed to a looser gel network. The oil globules of the $W_1/O/W_2$ emulsion disrupt the yogurt structure leading to softer products. Also, the texture (smoothness) of yogurt was shown to be affected in the presence of free or encapsulated probiotics (Kailasapathy, 2006). Adhesiveness is defined as the work necessary to pull the probe away from the food sample (i.e. the negative force) (Akgun *et al.*, 2016). Yogurts with $W_1/O/W_2$ emulsion and/or probiotic had lower adhesiveness values. Lower adhesiveness values are associated with high acceptability for yogurt like products (Mishra and Mishra, 2013). The increased adhesiveness values of the yogurts containing the $W_1/O/W_2$ emulsion can also be attributed to the presence of the oil since higher adhesive values have been recorded for yogurts with reduced fat content (Domagala *et al.*, 2005). The presence

of $W_1/O/W_2$ emulsion in the yogurt decreased its cohesiveness, which depends on the strength of internal bonds making up the body of the product (Kumar and Mishra, 2003). Considering that the nature of the protein matrix and the dispersion of fat influence the cohesiveness of the yogurt (Kumar and Mishra, 2003), the distribution of the emulsion and consequently the oil globules within the yogurt structure probably resulted in the observed lower cohesiveness values of the yogurts containing $W_1/O/W_2$ emulsion. Gumminess, the product of hardness and cohesiveness, expresses the energy required to disintegrate a semi-solid food product into a state ready for swallowing (Akgun *et al.*, 2016). Yogurt containing $W_1/O/W_2$ emulsion had lower gumminess values which can also be attributed to the oil phase of the emulsion (Kumar and Mishra, 2003).

7.5 Conclusions

For the first time, $W_1/O/W_2$ emulsion has been incorporated within set-type yogurt. The results show that $W_1/O/W_2$ emulsion can be successfully incorporated during acidification of milk without causing major disturbance to the yogurt structure. Although differences in viscosity, water retention capacity, acidification parameters and texture profiles were evident, however, the final product had similar physicochemical characteristics to those of the yogurt without $W_1/O/W_2$ emulsion. The presence of $W_1/O/W_2$ emulsion did not affect the growth kinetics of the bacteria. The oil globules of the $W_1/O/W_2$ emulsion maintained their inner W_1 phase and *L. paracasei* probiotic was present within the oil globules with relatively high viability. In this chapter an effective delivery dairy-based system for probiotics was developed which can be of interest for the dairy industry. However, more work is needed to understand the stability of yogurt with $W_1/O/W_2$ emulsion (e.g. over storage). Also, it would be of importance to test the survival of *L. paracasei* encapsulated in $W_1/O/W_2$

emulsion in a gastric-bile fluid to simulate stomach conditions. Nevertheless, the results of the present study seemed to be a promising attempt towards the development of functional dairy-based foods. In this chapter, the work was based on sunflower oil and surfactants based systems which would not be directly compatible with yogurt. However, this is the first study demonstrating the concept of incorporating $W_1/O/W_2$ emulsion in set-type yogurt and investigating its effect on the physicochemical properties and microbial responses. Furthermore, it is now concluded that in the future it would be worth to progress this further by expanding to a fully dairy and non-surfactant based systems.

CHAPTER 8: CONCLUSION

Nano-emulsions with droplet of average diameter ranging from 200-600 nm possess no antimicrobial properties against bacteria. The principal aim of this work was to assist expanding microbiological applications in complex emulsions (i.e. nano and double) by providing insight to interactions between microorganisms and emulsions. Previously, studies suggested that reducing the droplet size to sub-micron scale results or enhance anti-microbial properties. In contrary, other studies showed that nano-emulsions have no effect on microorganisms. Based on the results in this study it could be concluded that nano-emulsions do not directly possess antimicrobial properties unless antimicrobial agents are incorporated. It is important to keep in mind that the smallest average droplet size tested in this study was 170nm in diameter [D (4, 3)]; it could be that damaging effects due to high surface tension commence at smaller droplet size. These findings could be of interest to food, pharmaceutical and cosmetic industry as nano-emulsions, although more stable manufacturing, is more expensive and time consuming compared to conventional emulsions.

The release mechanism of bacteria from $W_1/O/W_2$ emulsion was due to oil globule bursting and can be controlled by altering its structure. The encapsulation of bacteria in $W_1/O/W_2$ emulsion has potential applications for the food industry from protecting probiotics to segregating bacterial species during fermentation to avoid antagonism. However, there is gap in knowledge about the mechanism by which bacteria are being released from the W_1 to W_2 phase in $W_1/O/W_2$ and how to modulate this release mechanism. This study provided insight on the mechanism of release of bacteria in $W_1/O/W_2$ emulsion. It was shown that this release can be affected by changing the structure of the $W_1/O/W_2$ emulsion. Even

though in this report the release of bacteria from $W_1/O/W_2$ emulsions can be modulated to a certain extent only, however, more work needs to be done in order to understand the release mechanism with other emulsifiers that are widely used in industry. Understanding how to control bacterial release from the W_1 to W_2 in $W_1/O/W_2$ emulsions can be useful for the food industry. For example, in a fermentation process, a secondary species of bacteria that is responsible for aroma and/or flavour of the final product can be released over time in a controlled manner. It may also be important to separate the starter culture and the secondary species to prevent any possible antagonistic effects between them. Having the secondary species encapsulated in $W_1/O/W_2$ emulsion can reduce the risk of contamination that may occur because of inoculating the secondary species during the fermentation process.

The incorporation of $W_1/O/W_2$ emulsion in set-type yogurt for the delivery of probiotic *L. paracasei* is feasible. After understanding the behaviour of $W_1/O/W_2$ emulsion in the presence of bacteria and to take it a further step forward an attempt to incorporate $W_1/O/W_2$ emulsion in a dairy-based food was made. The $W_1/O/W_2$ emulsion was successfully incorporated within the set-style yogurts' structure. Although the $W_1/O/W_2$ emulsion had a significant effect on the texture and physicochemical properties of the yogurt there was no effect on bacterial growth kinetics and probiotics had high viability and emulsion was stable at the end of the acidification process. It is important to consider if these textural and physicochemical differences are also affecting the sensory properties of the yogurt. These findings are of importance to the dairy industry since the incorporation of $W_1/O/W_2$ emulsion in set-style yogurt can be used to reduce fat content and salt, enhance flavour, and protect and deliver probiotics.

CHAPTER 9: FUTURE WORK

Based on the findings from this report, further work can be done in the following areas:

- **The droplet size of the nano-emulsion may have not been small enough to see the observed antimicrobial activity reported in literature.** The nano-emulsions with the smallest droplet size in this report had an average droplet size of 170nm. To extend the conclusions made regarding the effect of droplet size on bacteria it would be interesting to work with nano-emulsions with a smaller average droplet size. This can be achieved by changing the formulation by matching the densities between the dispersed and continuous phase or increasing the hydrophilic surfactant concentration.
- **Interactions between bacteria and $W_1/O/W_2$ emulsions were investigated in the presence of low molecular weight surfactants.** In this report, only two low-molecular weight surfactants were used, the lipophilic PGPR and the hydrophilic Tween80. To extend the conclusions made regarding the release mechanism of bacteria from $W_1/O/W_2$ emulsions it would be interesting to work with other surfactants, for example macromolecular surfactants (e.g. polymeric surfactants or proteins).
- **All the emulsions had a broad droplet size distribution.** In this report, a rotor-stator mixer (Silverson) was used to form the emulsions which achieves a wide range of droplet size distribution. To better understand the release mechanism of bacteria from $W_1/O/W_2$ emulsion it would be important to obtain a narrow range of droplet size distribution and this can be achieved by using membrane emulsification or microfluidics.

- **Osmotic balance alteration was achieved by testing a limited range of NaCl concentrations.** Regarding the work done on controlling the release of bacteria from $W_1/O/W_2$ emulsion globules under hyper-osmotic pressure it would be better investigating the release with a wider range of NaCl concentration starting from <0.04 M as beyond that concentration the release seems to reach its maximum. The viscoelasticity of the $W_1/O/W_2$ emulsions could be measured to get a better understanding of the effects of NaCl and osmotic balance alteration on the strength of the interfacial film. Also, it would be interesting to study the release mechanism of bacteria from $W_1/O/W_2$ emulsion as a function of droplet size.
- **The coarse emulsions had poor creaming stability.** To a certain extent food-based emulsion are stable against creaming. In this report, no attempt was done to prevent creaming. For the results in this report to be of interest to the food industry it would be important to stabilise the emulsions against creaming by increasing the viscosity of the continuous phase and/or increasing the density of the inner W_1 phase.
- **Creaming behaviour was assessed based on observation only.** In this report changes in creaming height or thickness was used to quantify the loss of $W_1/O/W_2$ emulsion globules due to the bursting phenomenon. Since the cream thickness changes with swelling and shrinking as well as instability phenomenon like flocculation or aggregation of droplets, therefore, a better method should be used to quantify the bursting mechanism (e.g. using a microscope or a Coulter counter).
- **The texture and physicochemical properties of set-type yogurt with $W_1/O/W_2$ emulsion was only tested until the end of fermentation.** In the

study investigating the incorporation of $W_1/O/W_2$ in set-type yogurt the physicochemical properties of the yogurt were only tested until the fermentation ends. To use the conclusions of this report in the food industry it would be necessary to monitor the stability of yogurt with $W_1/O/W_2$ emulsions over a longer period and conduct sensory analysis and consumer tests. Also, the stability of the $W_1/O/W_2$ emulsion was only qualitatively assessed and a quantitative method is required to better understand how the stability of the emulsion may be altered during the acidification process. Also, it would be interesting to test the survival of the encapsulated *L. paracasei* in a gastric juice model.

- **The stability of the $W_1/O/W_2$ emulsion in the acidifying milk was monitored by observation using microscopic techniques.** The stability of the $W_1/O/W_2$ emulsion within the yogurt was monitored using qualitative methods (i.e. microscopic observation) and it would be more valuable to include a quantitative method as well (e.g. oil globule size distribution). This was considered during the study but due to lack of time it was not possible to achieve this as it requires obtaining a large amount of imaging and further processing which is time consuming. Although techniques based on light scattering are fast, but we found it to be unsuitable for this study due to the overlapping between the size distribution of the emulsion and the aggregated fat droplets of the acidifying yogurt (data not shown).
- **The viability of encapsulated probiotic *L. paracasei* in set-type yogurt was only monitored until the end of fermentation.** The encapsulated probiotic within $W_1/O/W_2$ emulsion maintained high viability throughout the acidification process. In this study, the viability of the probiotic was only assessed after the end of the acidification process and since yogurt in the market is stored at

chilled temperature for weeks or even months. Therefore, it would be useful to investigate the viability of the probiotic during storage at refrigeration temperatures.

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APPENDICES

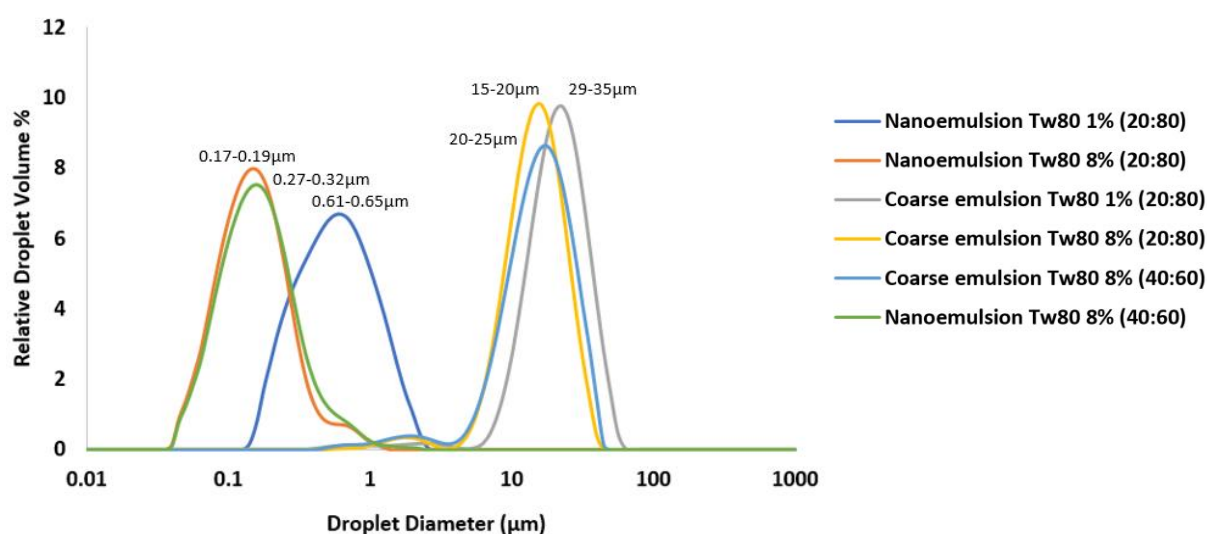


Figure A1 The diameter size (μm) of the oil droplets measured by light scattering [D (4, 3)] of freshly made O/W nano-emulsion and coarse emulsion. The O/W emulsions were prepared with 20% or 40% oil phase stabilised with 1% or 8% Tween80 in the continuous phase (M9 minimal growth medium).

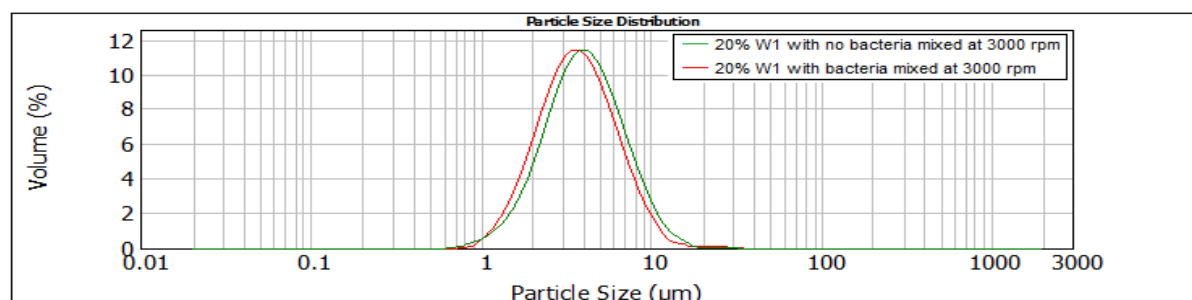


Figure A2 Particle size [D (4, 3)] analysis of the W_1 droplets from W_1/O emulsion. W_1/O emulsion is prepared with 20% W_1 with or without bacteria stabilised with 2% lipophilic surfactant. Measurements were taken at refractive index of 1.33.

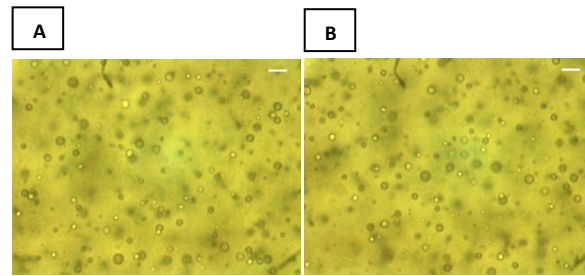


Figure A3 Optical images of W_1/O emulsions. W_1/O emulsions were prepared with water (W_1) volume percentage of 20% with (A) or without (B) bacteria and stabilised with 2% PGPR. Scale bar: 5 μ m.

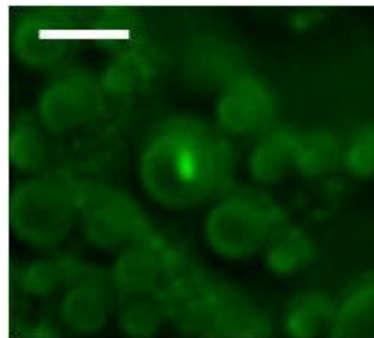
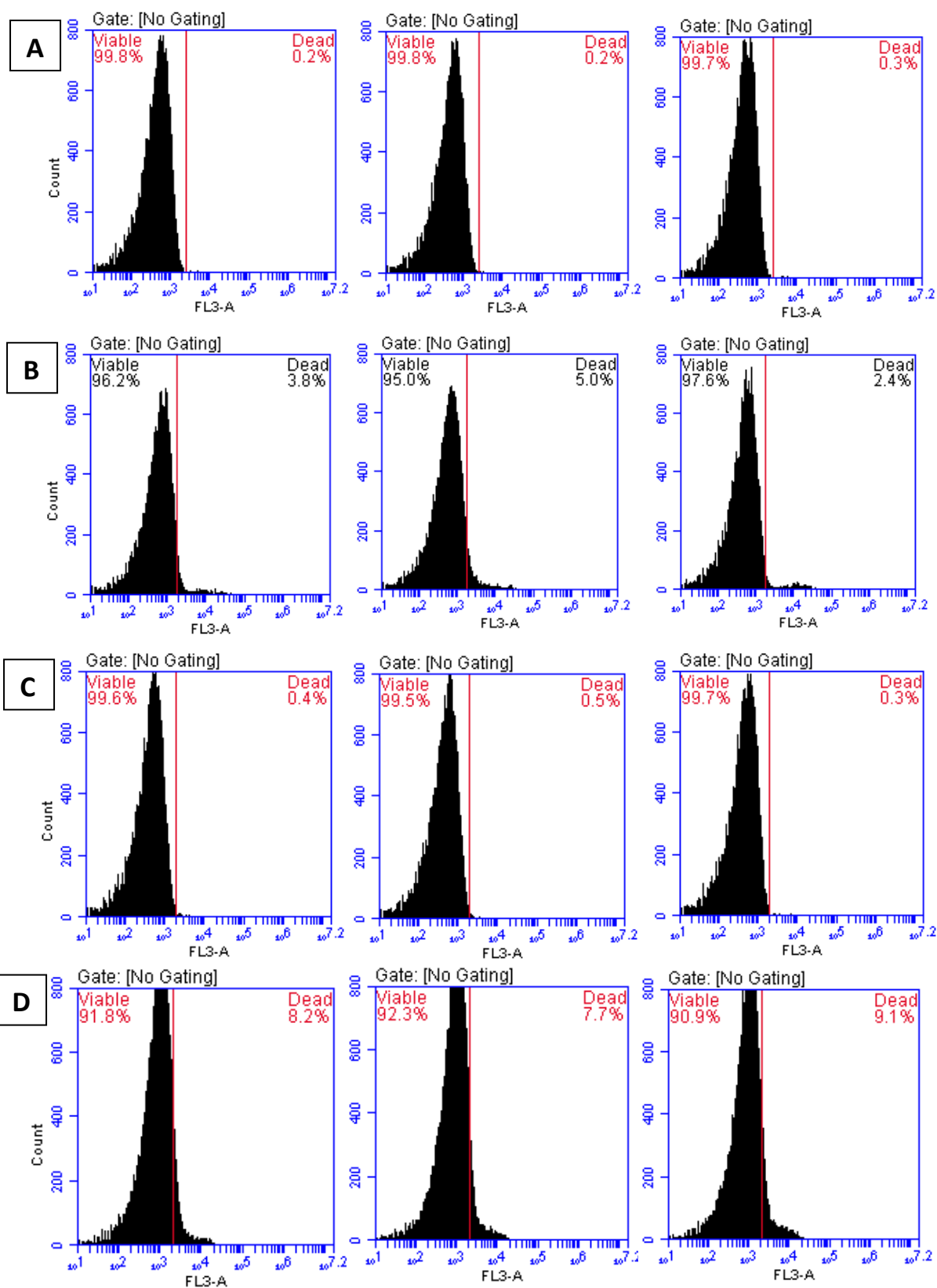


Figure A4 Fluorescent images of W_1 droplets encapsulating *E. coli*-GFP within the primary W_1/O emulsion. Scale bar: 5 μ m.



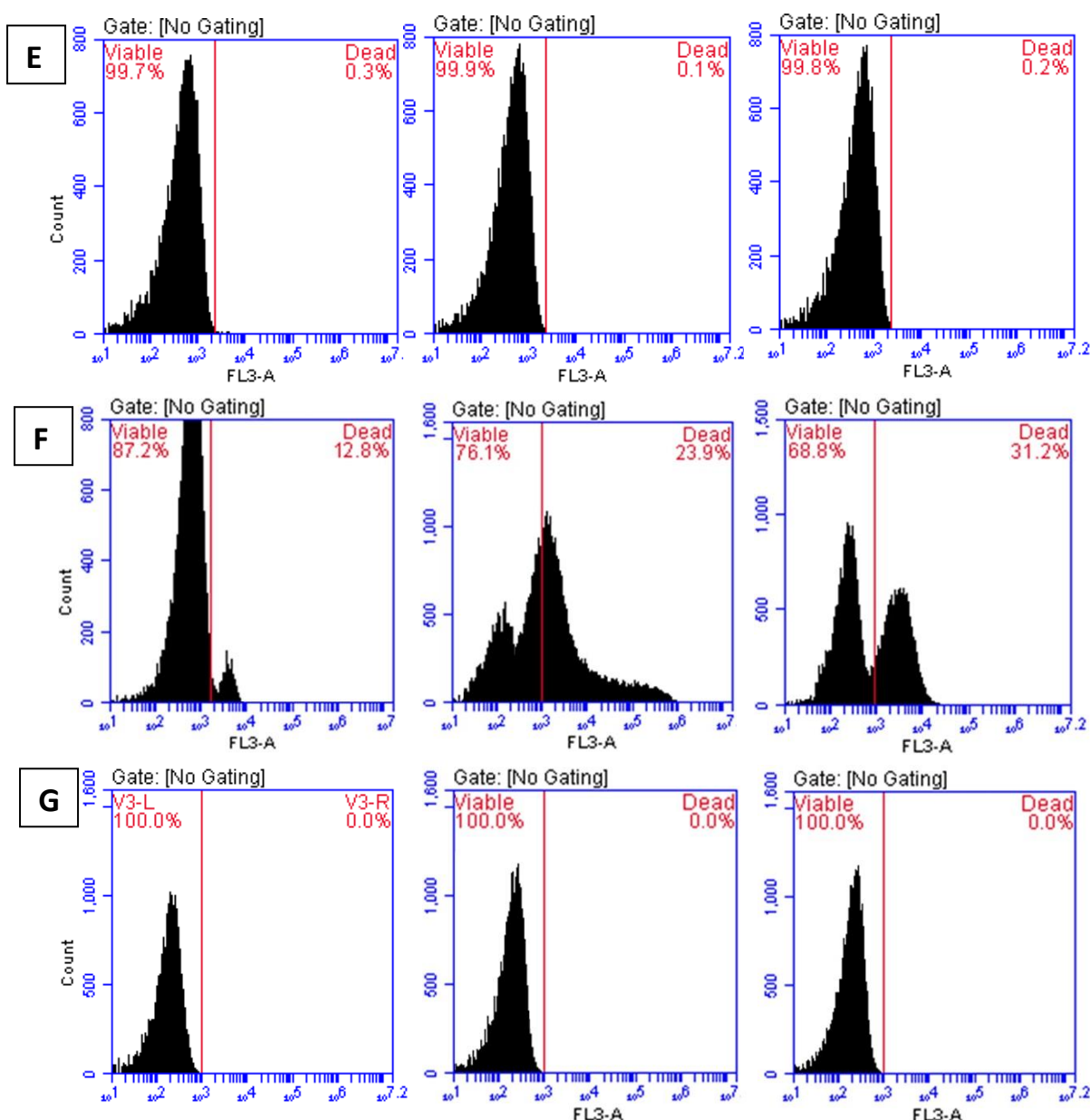


Figure A5 Representative flow cytometry scatter plots showing the percentage of PI-positive (a) *E. coli* (MG1655), (b) *E. coli* (BW25113), (c) *E. coli* (JM109), (d) *E. coli* (MC4100), (e) *E. coli* (PHL644), (f) *B. cereus*, (g) *S. epidermidis*, cells in M9 medium (control), nano-emulsion or coarse emulsion at day 7 incubated at 25°C (similar data on 0, 2 days not shown). The O/W emulsions were prepared with 40% oil phase and stabilised with 8% Tween80 in the continuous phase (M9 minimal growth medium).

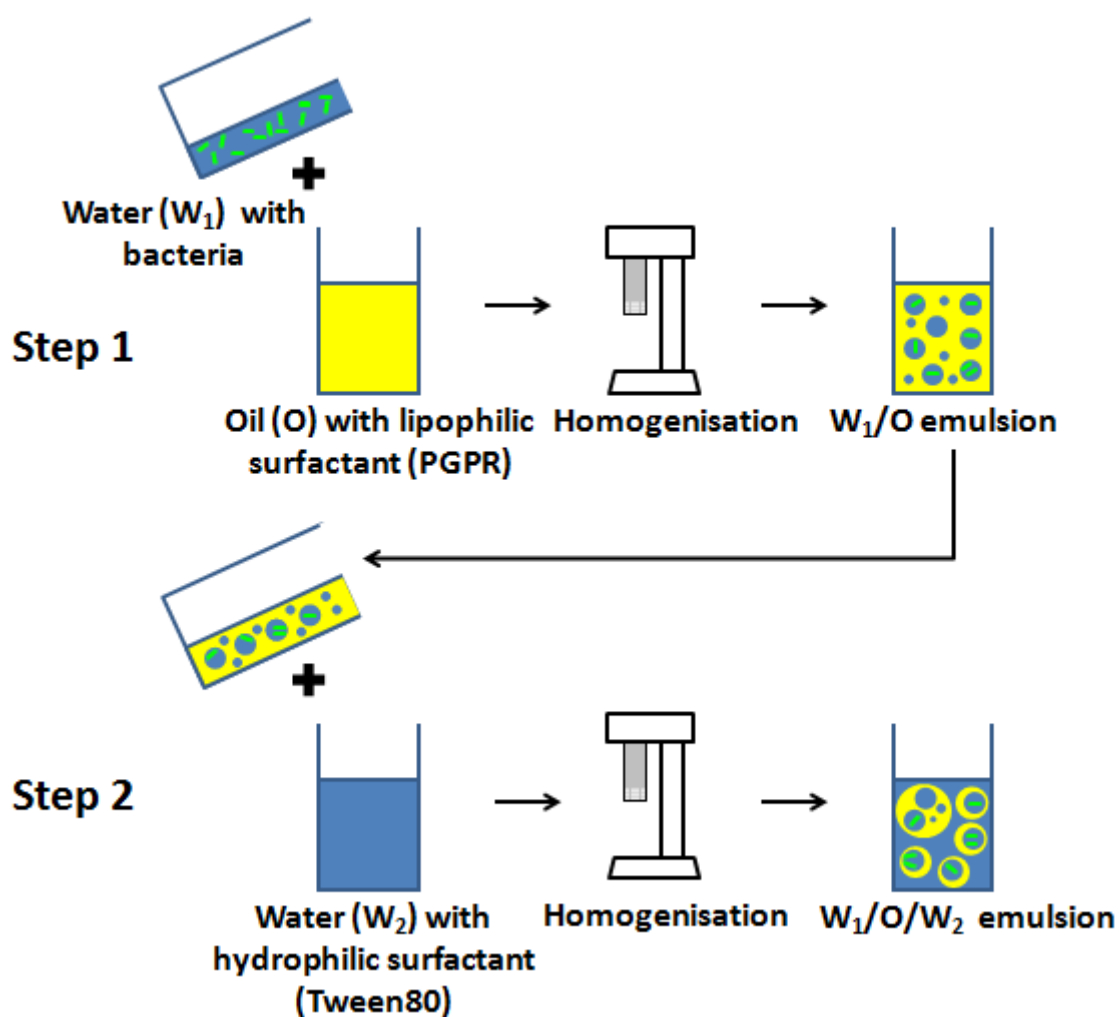


Figure A6 Schematic illustration of the two-step emulsification of $W_1/O/W_2$ emulsion and encapsulation of bacteria.

Table A1 The rotational speeds of the rotor used to homogenize the different formulations of W_1/O and $W_1/O/W_2$.

W/O_1 percentage	Tween80 percentage	Rotor speed (rpm) for W/O_1	Rotor speed (rpm) for $W_1/O/W_2$
20	1	3000	2000
20	5	3000	1800
40	1	5000	2300
40	5	5000	2600

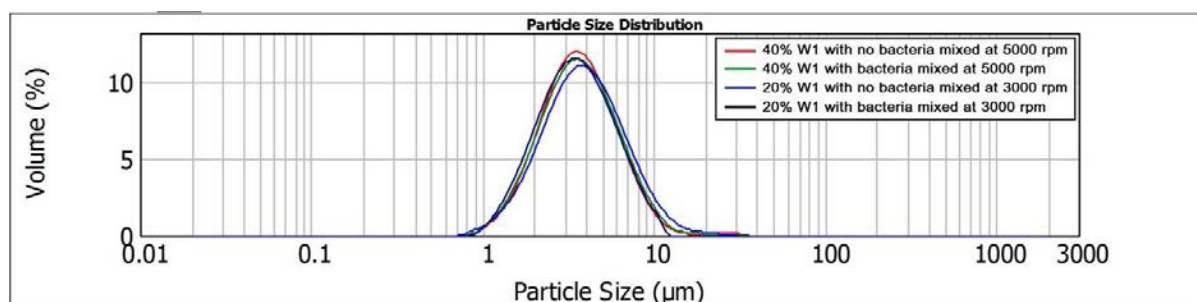


Figure A7 Particle size [D (4, 3)] analysis of the W_1 droplets from W_1/O emulsion. W_1/O emulsion is prepared with 20% or 40% W_1 with or without bacteria stabilised with 2% lipophilic surfactant. Measurements were taken at refractive index of $n_D^{22}1.33$.

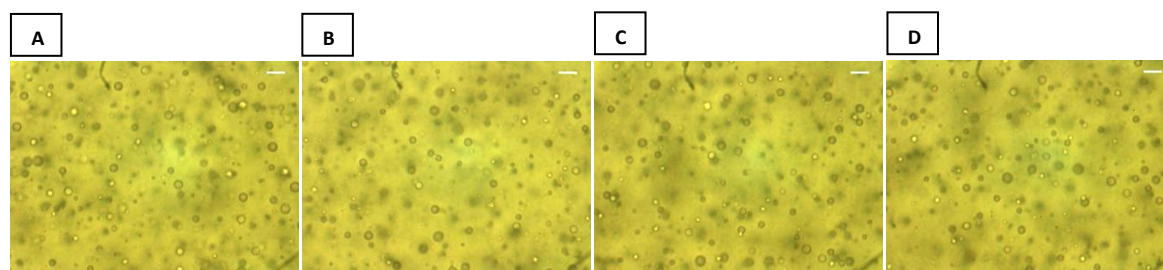


Figure A8 Optical images of W_1/O emulsions. W_1/O emulsions were prepared with water (W_1) volume percentage of 20% or 40% with or without bacteria and stabilised with 2% PGPR. The formulations were as follows: A) 40% W_1 with no bacteria mixed at 5000rpm; B) 40% W_1 with bacteria mixed at 5000 rpm; C) 20% W_1 with no bacteria mixed at 3000 rpm; D) 20% W_1 with bacteria mixed at 3000rpm. Scale bar: 10 μ m.

Table A2 The encapsulation efficiency (%) of *E. coli*-GFP and D (4, 3) of different $W_1/O/W_2$ emulsions. Results are taken from a minimum of 3 independent experiments.

DE Formulations	20% W_1 , 1% Tw80	20% W_1 , 5% Tw80	40% W_1 , 1% Tw80	40% W_1 , 5% Tw80
Encapsulation efficiency (%)	99.95 \pm 0.02	99.90 \pm 0.03	99.93 \pm 0.02	99.90 \pm 0.09
D (4, 3)	67.00 \pm 1.37	64.10 \pm 0.85	70.90 \pm 0.17	70.01 \pm 1.00

Video A1 Optical video-microscopy showing oil globule bursting and release of W_1 droplets into W_2 after adding NaCl in W_2 . The $W_1/O/W_2$ emulsion was prepared with inner W_1 phase volume percentage of 40% containing bacteria and stabilised with 1% Tween80 containing 0.085 M NaCl in the W_2 phase.

Video A2 Fluorescence video-microscopy showing bursting of oil globule and release of *E. coli*-GFP after altering the osmotic balance. The double emulsion was prepared with inner W_1 phase volume percentage of 40% containing bacteria and stabilised with 1% Tween80 containing 0.085 M NaCl in the W_2 phase.

Video A3 Fluorescence video-microscopy showing *E. coli*-GFP cells located within oil globules after loss of W_1 phase with no bursting of the oil globules occurring after osmotic balance alteration. The $W_1/O/W_2$ emulsion was prepared with inner W_1 phase volume percentage of 20% containing bacteria and stabilised with 5% Tween 80 containing 0.085 M NaCl in the W_2 phase.

Video A4 Fluorescence video-microscopy showing *E. coli*-GFP cells located within two oil globules before and after coalescing with each other during loss of W_1 phase due to the presence of NaCl in W_2 . The $W_1/O/W_2$ emulsion was prepared with inner W_1 phase volume percentage of 40% containing bacteria and stabilised with 1% Tween 80 containing 0.085 M NaCl in the W_2 phase.

Video A5 Fluorescence video-microscopy showing bursting of oil globule and release of *E. coli*-GFP after altering the osmotic balance by injecting in the $W_1/O/W_2$ emulsion in a hypo-osmotic solution. The $W_1/O/W_2$ emulsion was prepared with inner W_1 phase volume percentage of 40% with 0.085 M NaCl containing *E. coli*-GFP and stabilised with 1% Tween80 in W_1 and 2% PGPR in the oil phase.

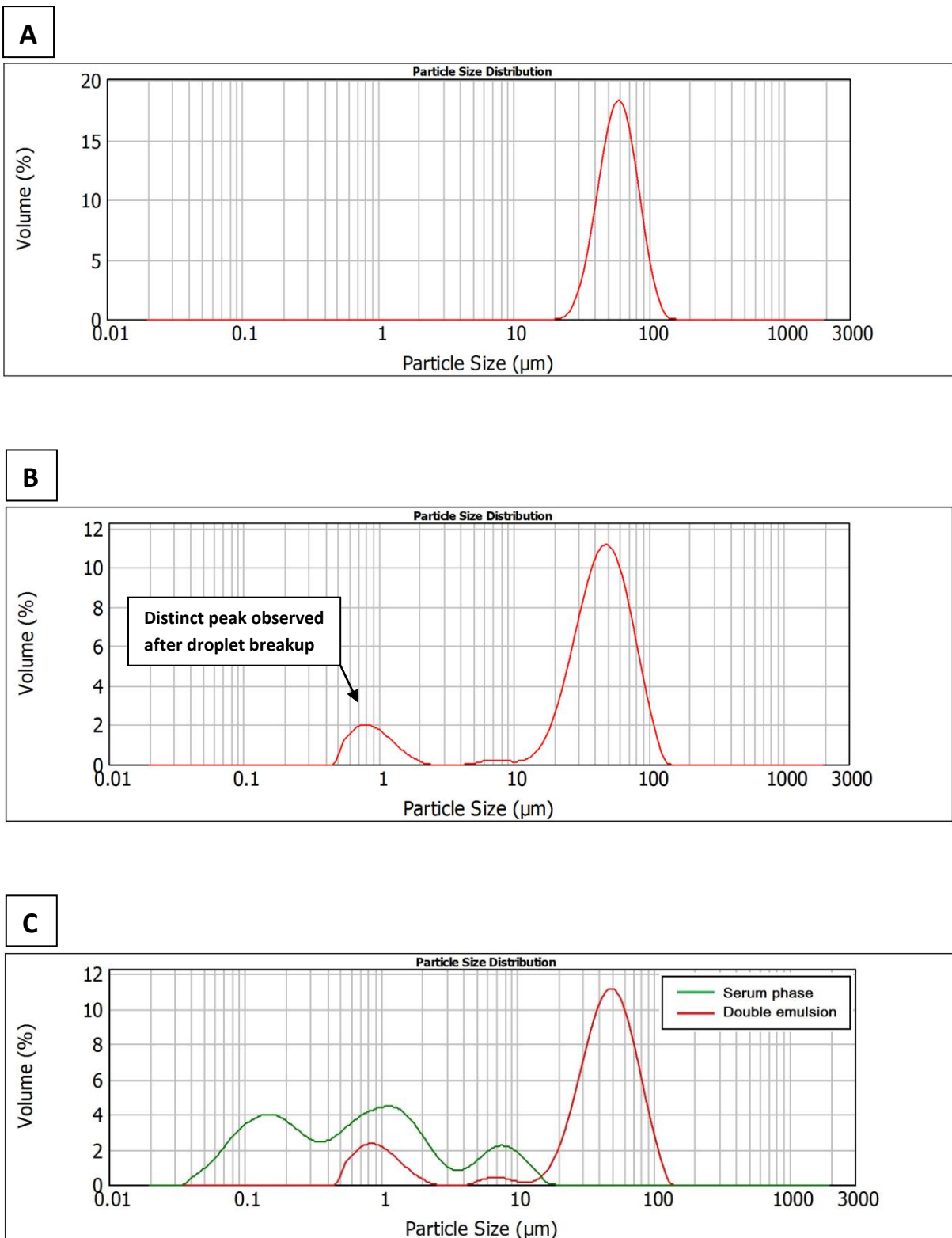


Figure A9 Particle size [D (4, 3)] analysis of (A) freshly made $W_1/O/W_2$ emulsion before altering the osmotic balance, (B) $W_1/O/W_2$ emulsion after altering the osmotic balance and of the serum phase of $W_1/O/W_2$ emulsion after altering the osmotic balance (C). The $W_1/O/W_2$ emulsions were prepared with 40% W_1 containing no bacteria and stabilised with 1% Tween80 and 2% PGPR. Osmotic balance alteration was achieved by adding NaCl (0.085 M) to the W_2 phase. Measurements were taken at refractive index of n_D^{22} 1.396.

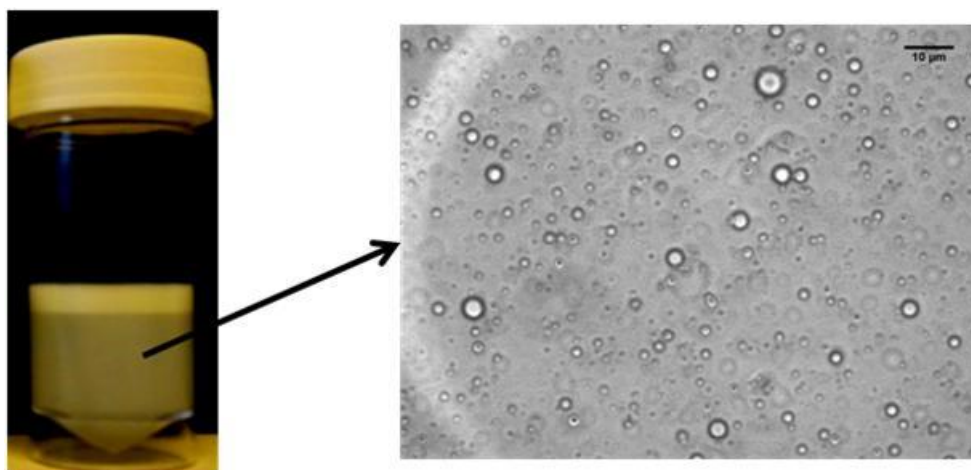


Figure A10 Optical observation of the serum phase from the $W_1/O/W_2$ emulsion after osmotic balance alteration. The W_1 droplets persisted in the serum phase during incubation. Scale bar: 10 μ m.

Table A3 The rotational speeds of the rotor used to homogenize the different formulations of primary W_1/O and double $W_1/O/W_2$ emulsion.

W_1/O percentage	Tween80 percentage	PGPR percentage	Rotor speed (rpm) for W_1/O	Rotor speed (rpm) for $W_1/O/W_2$
20	1	2	3000	2000
20	5	2	3000	1800
40	1	2	5000	2700
40	5	2	5000	2300
40	1	4	2000	2700
40	1	6	1500	2700
40	1	8	1200	2700
40	0.5	2	5000	2900
40	10	2	5000	2000

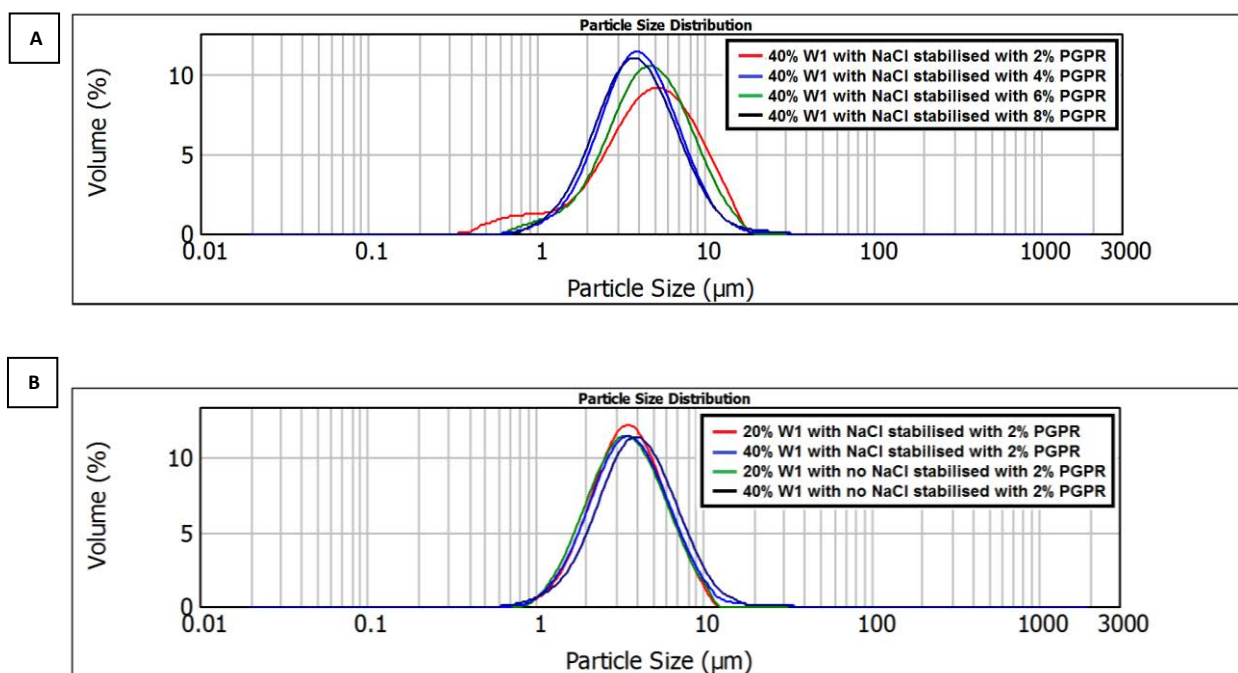


Figure A11 Particle size [D (4, 3)] analysis of the W_1 droplets from primary W_1/O emulsions. The formulations were as follows: A) W_1/O emulsion was prepared with 20% or 40% W_1 with or without bacteria stabilised with 2% PGPR. Measurements were taken at refractive index of n_D^{22} 1.33; B) W_1/O emulsion was prepared with 20% or 40% W_1 with or without bacteria stabilised with 2% PGPR. Measurements were taken at refractive index of n_D^{22} 1.33.

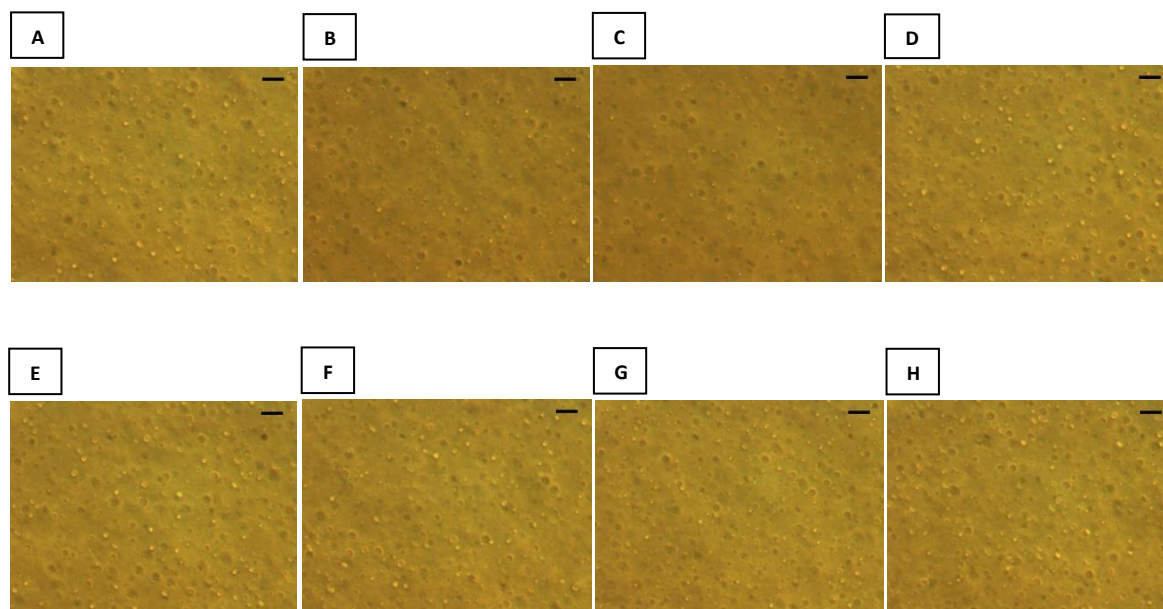


Figure A12 Optical images of primary W_1/O emulsions. Primary W_1/O emulsions were prepared with water (W_1) volume percentage of 20% or 40% with or without bacteria and stabilised with 2% PGPR. The formulations were as follows: A) 40% W_1 with 0.085 M NaCl stabilised with 2% PGPR; B) 40% W_1 with 0.085 M NaCl stabilised with 4% PGPR; C) 40% W_1 with 0.085 M NaCl stabilised with 6% PGPR; D) 40% W_1 with 0.085 M NaCl stabilised with 8% PGPR; E) 20% W_1 with 0.085 M NaCl stabilised with 2% PGPR; F) 20% W_1 with 0.085 M NaCl stabilised with 2% PGPR; G) 20% W_1 with no NaCl stabilised with 2% PGPR; F) 40% W_1 with no NaCl stabilised with 2% PGPR. Scale bar: 10 μ m.

Table A4 The encapsulation efficiency (%) of *E. coli*-GFP and D (4, 3) of different $W_1/O/W_2$ emulsions. Results show means \pm standard deviation taken from a minimum of 3 independent experiments.

Formulations	Encapsulation efficiency (%)	D (4,3)
40% W_1 , 1% Tw80, 2% PGPR (NaCl)	99.9 \pm 0.03	70.6 \pm 5.41
40% W_1 , 1% Tw80, 4% PGPR (NaCl)	99.8 \pm 0.06	71.6 \pm 3.60
40% W_1 , 1% Tw80, 6% PGPR (NaCl)	99.9 \pm 0.03	71.9 \pm 4.51
40% W_1 , 1% Tw80, 8% PGPR (NaCl)	99.8 \pm 0.03	72.6 \pm 4.43
40% W_1 , 0.5% Tw80, 2% PGPR (NaCl)	99.8 \pm 0.01	66.6 \pm 0.90
40% W_1 , 5% Tw80, 2% PGPR (NaCl)	99.9 \pm 0.05	69.6 \pm 3.71
40% W_1 , 10% Tw80, 2% PGPR (NaCl)	99.8 \pm 0.05	73.6 \pm 1.30
20% W_1 , 1% Tw80, 2% PGPR (NaCl)	99.9 \pm 0.01	67 \pm 0.21
20% W_1 , 5% Tw80, 2% PGPR (NaCl)	99.8 \pm 0.16	66.9 \pm 0.10
20% W_1 , 1% Tw80, 2% PGPR (water)	99.9 \pm 0.02	70.9 \pm 4.00
20% W_1 , 5% Tw80, 2% PGPR (water)	99.8 \pm 0.03	70.5 \pm 5.21
40% W_1 , 1% Tw80, 2% PGPR (water)	99.9 \pm 0.06	71.5 \pm 0.40
40% W_1 , 5% Tw80, 2% PGPR (water)	99.8 \pm 0.02	70 \pm 2.72

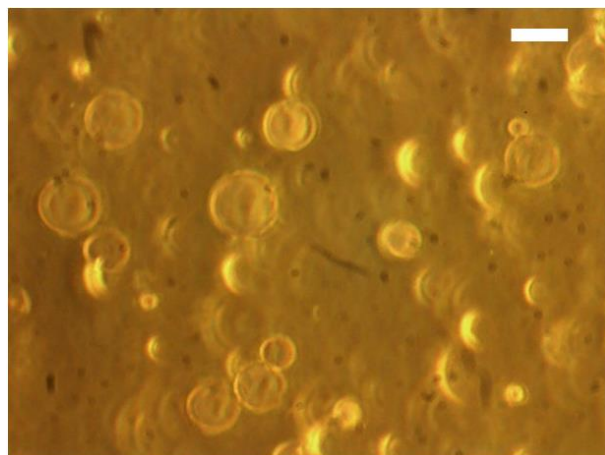


Figure A13 Optical images of primary W_1/O emulsions. Primary W_1/O emulsions were prepared with milk (W_1) volume percentage of 40% with or without *L. paracasei* probiotic and stabilised with 2% PGPR. Scale bar: 10 μ m.

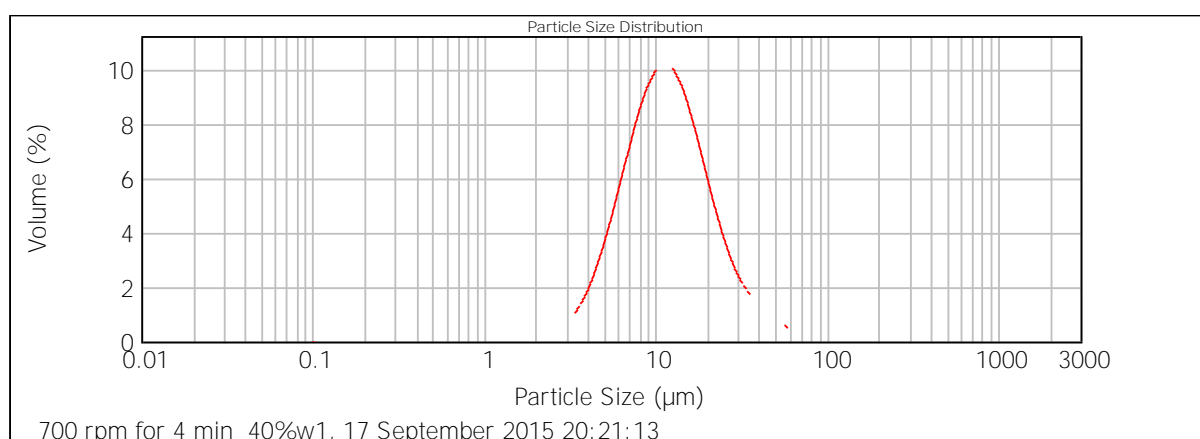


Figure A14 Particle size [D (4, 3)] analysis of the W_1 droplets from freshly made primary W_1/O emulsion. Measurements were taken at refractive index of n_D^{22} 1.396.

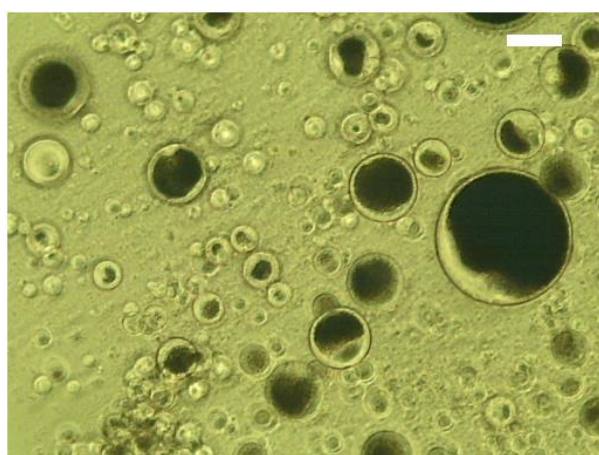


Figure A15 Optical images of primary $W_1/O/ W_2$ emulsions. $W_1/O/ W_2$ emulsions were prepared with milk (W_1) volume percentage of 40% with or without *L. paracasei* probiotic stabilised with 2% PGPR. The continuous W_2 phase was milk. Scale bar: 100 μ m.

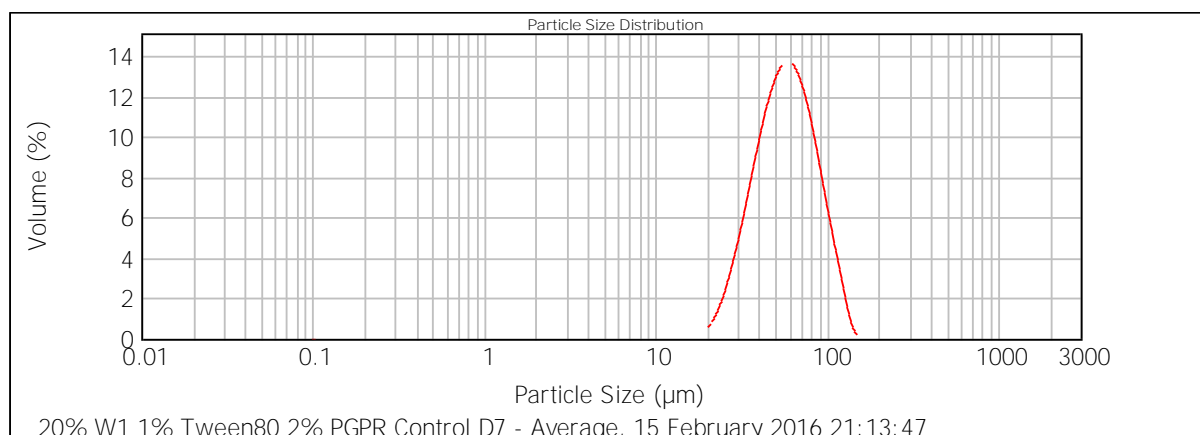


Figure A16 Particle size [D (4, 3)] analysis of the oil globules in freshly made W₁/O/W₂ emulsion. Measurements were taken at refractive index of n_b^{22} 1.396.

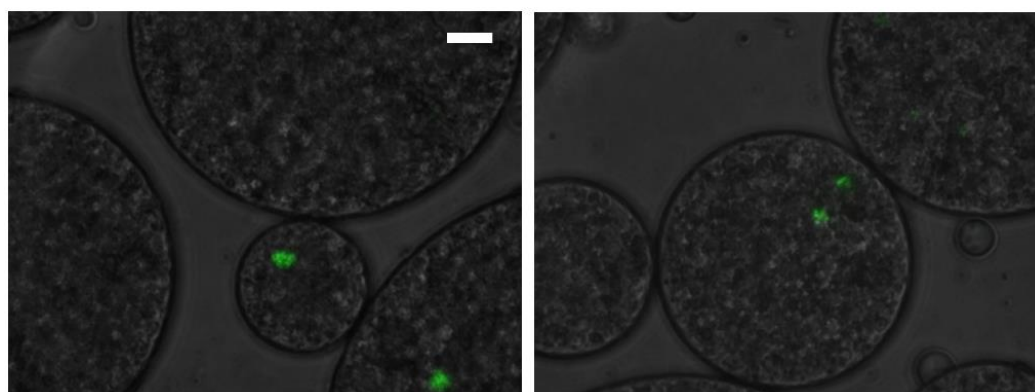


Figure A17 Fluorescent images of W₁/O/W₂ emulsion globules encapsulating *L. paracasei* probiotic (green). Scale bar: 10 μm .

Table A5 Viscosity results during acidification process of milk with and without the addition of W₁/O/W₂ emulsion (DE) and/or *L. paracasei* (probiotic). Results show mean \pm SEM taken from a minimum of 3 independent experiments. Mean values with different letters are significantly different ($P < 0.05$). The data was analysed with one-way ANOVA. The viscosity (mPas.s), consistency constant K and power-law index n for each sample was measured over a shear rate range 700-1000 s^{-1} .

Time (min)	Yogurt			Yogurt with free probiotics			Yogurt with DE			Yogurt encapsulated probiotics		
	Viscosity (Pa.s)	K	n	Viscosity (Pa.s)	K	n	Viscosity (Pa.s)	K	n	Viscosity (Pa.s)	K	n
0	9.93 \pm 1.00 ^a	9.10	0.01	9.93 \pm 1.00 ^a	9.10	-0.01	9.93 \pm 1.00 ^a	9.10	-0.01	9.93 \pm 1.00 ^a	9.10	-0.01
60	11.97 \pm 0.29 ^a	0.64	0.41	12.02 \pm 0.13 ^a	1.18	0.31	10.38 \pm 0.26 ^b	1.29	0.28	10.49 \pm 0.57 ^b	1.34	0.27
120	11.95 \pm 0.95 ^a	0.97	0.38	15.63 \pm 0.80 ^c	0.01	0.87	19.02 \pm 0.87 ^b	0.88	0.41	19.81 \pm 0.35 ^b	0.77	0.46
180	35.38 \pm 3.67 ^a	0.33	0.65	32.11 \pm 1.34 ^a	0.19	0.73	36.47 \pm 1.06 ^a	0.01	0.83	31.56 \pm 1.18 ^a	0.04	0.96
240	38.09 \pm 3.53 ^a	0.33	0.70	41.46 \pm 1.78 ^a	0.39	0.69	29.02 \pm 2.16 ^b	0.03	0.95	18.69 \pm 1.37 ^b	0.37	0.56
300	42.06 \pm 3.91 ^a	12.01	0.16	45.51 \pm 1.13 ^a	16.78	0.10	36.39 \pm 1.93 ^b	0.17	0.75	27.21 \pm 1.79 ^b	0.14	0.76



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Do oil-in-water (O/W) nano-emulsions have an effect on survival and growth of bacteria?

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ABSTRACT

Nano-emulsions (typically droplet diameter < 1 µm) are common in foods, and have been extensively reported to present antimicrobial activity, however, the mechanism is not well defined, and some studies reported no effect. A review of the literature was conducted and revealed strongly contradictory reports regarding the antimicrobial effect of nano-emulsions even in reference to similar microbial species and formulations. Following up, this study aimed to investigate the effect of nano-emulsions on four bacterial species (*Staphylococcus epidermidis*, *Bacillus cereus*, *Lactobacillus acidophilus* and five *Escherichia coli* strains) possessing different surface charge and hydrophobicity. Model oil-in-water (O/W) emulsions with different size of oil droplets were prepared with sunflower oil stabilised by polysorbate 80 (Tween80) emulsifier (hydrophilic), using high shear mixing followed by ultrasonication. The viability of bacteria was monitored by culture, membrane integrity was assessed with flow cytometric analysis with propidium iodide (PI) staining and fluorescence microscopy monitored the spatial distribution of cells within the O/W emulsions. The stability of the nano-O/W emulsions in the presence of bacteria was assessed by monitoring the droplet size [D (4, 3)] and creaming height. In contrast to other reports the survival and growth of bacteria was not affected by the size of the oil droplets, no damage to the bacterial membrane was evident with flow cytometry and emulsion stability was not affected by the presence of bacteria during 7 days of storage. Furthermore, the antimicrobial activity of caprylic acid (CA) was compared between O/W coarse and nano-emulsions while varying the concentration of the hydrophilic surfactant Tween80. The activity of CA was similar in nano-emulsion and coarse emulsion; however, it was higher than in bulk oil and was reduced with increasing Tween80 concentration, suggesting that its efficacy is dictated by formulation rather than oil droplet size. The results demonstrated no enhanced antimicrobial activity due to nano-sized oil droplets and that conclusions on nano-emulsions should be taken with caution.

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The first part of the paper discusses the importance of the research and the objectives of the study. It then presents a literature review of the existing research on the topic. The second part of the paper describes the methodology used in the study, including the data collection and analysis techniques. The third part of the paper presents the results of the study, and the fourth part discusses the conclusions and implications of the findings.

The study was conducted using a quantitative research design. Data was collected from a sample of 100 participants, and the results were analyzed using statistical software. The findings of the study indicate that there is a significant relationship between the variables being studied.

The results of the study suggest that the research has important implications for the field. Further research is needed to explore the relationship between the variables in more detail.

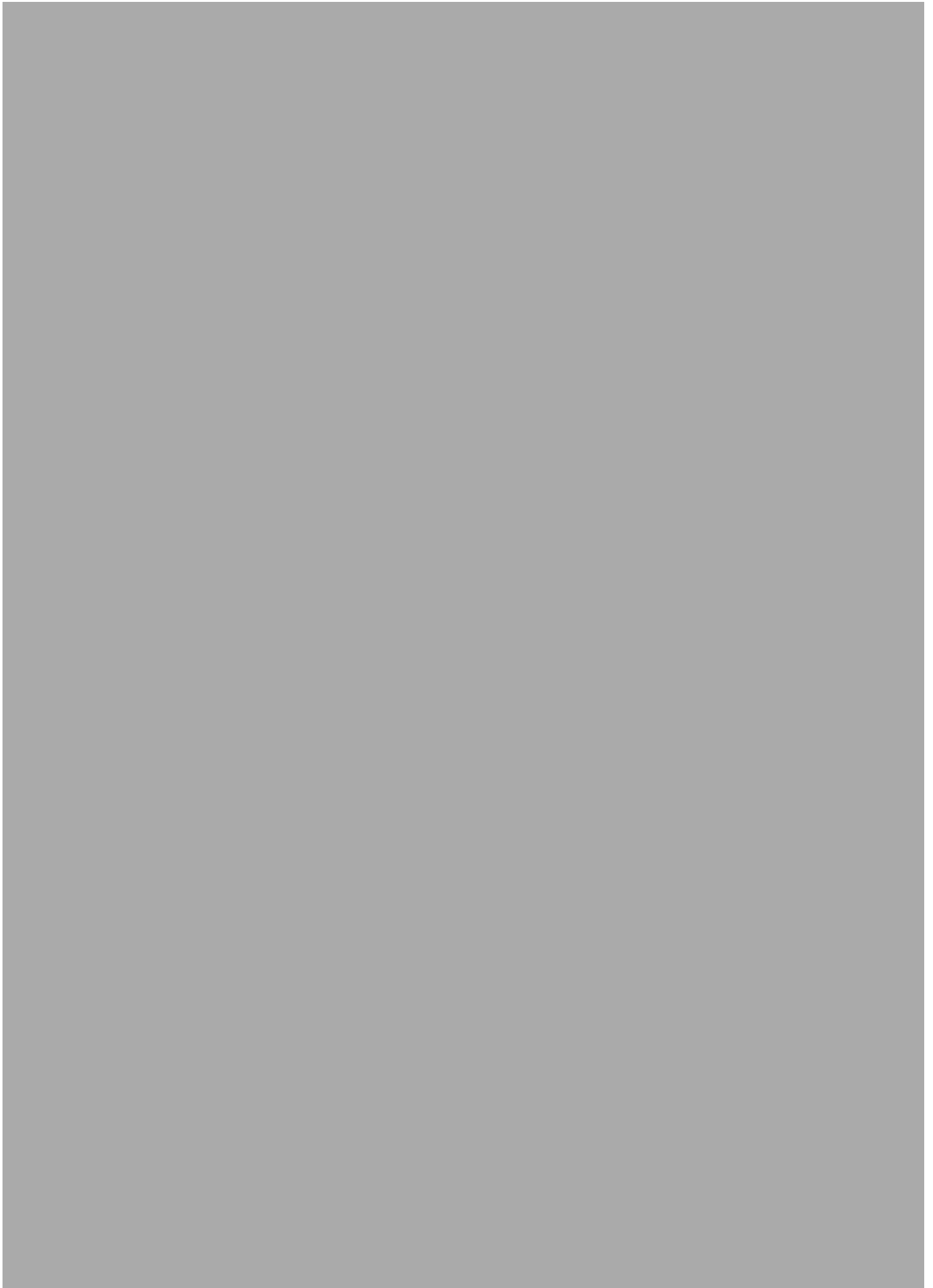
In conclusion, the study has provided valuable insights into the topic and has contributed to the existing body of knowledge. The findings have important implications for the field and suggest that further research is needed.











the 1990s, the number of people in the UK who are employed in the public sector has increased by 1.5 million (1990–1999) and the number of people in the public sector has increased by 2.5 million (1990–1999) (Department of Health 2000).

There is a growing emphasis on the need to improve the efficiency of the public sector. The Department of Health (2000) has set out a number of targets for the public sector, including a 10% reduction in the number of people employed in the public sector by 2005. This has led to a number of initiatives to improve the efficiency of the public sector, including the introduction of the 'New Deal' for the public sector.

The 'New Deal' for the public sector is a set of initiatives designed to improve the efficiency of the public sector. It includes a number of measures to reduce the number of people employed in the public sector, including the introduction of the 'New Deal' for the public sector. The 'New Deal' for the public sector is a set of initiatives designed to improve the efficiency of the public sector.

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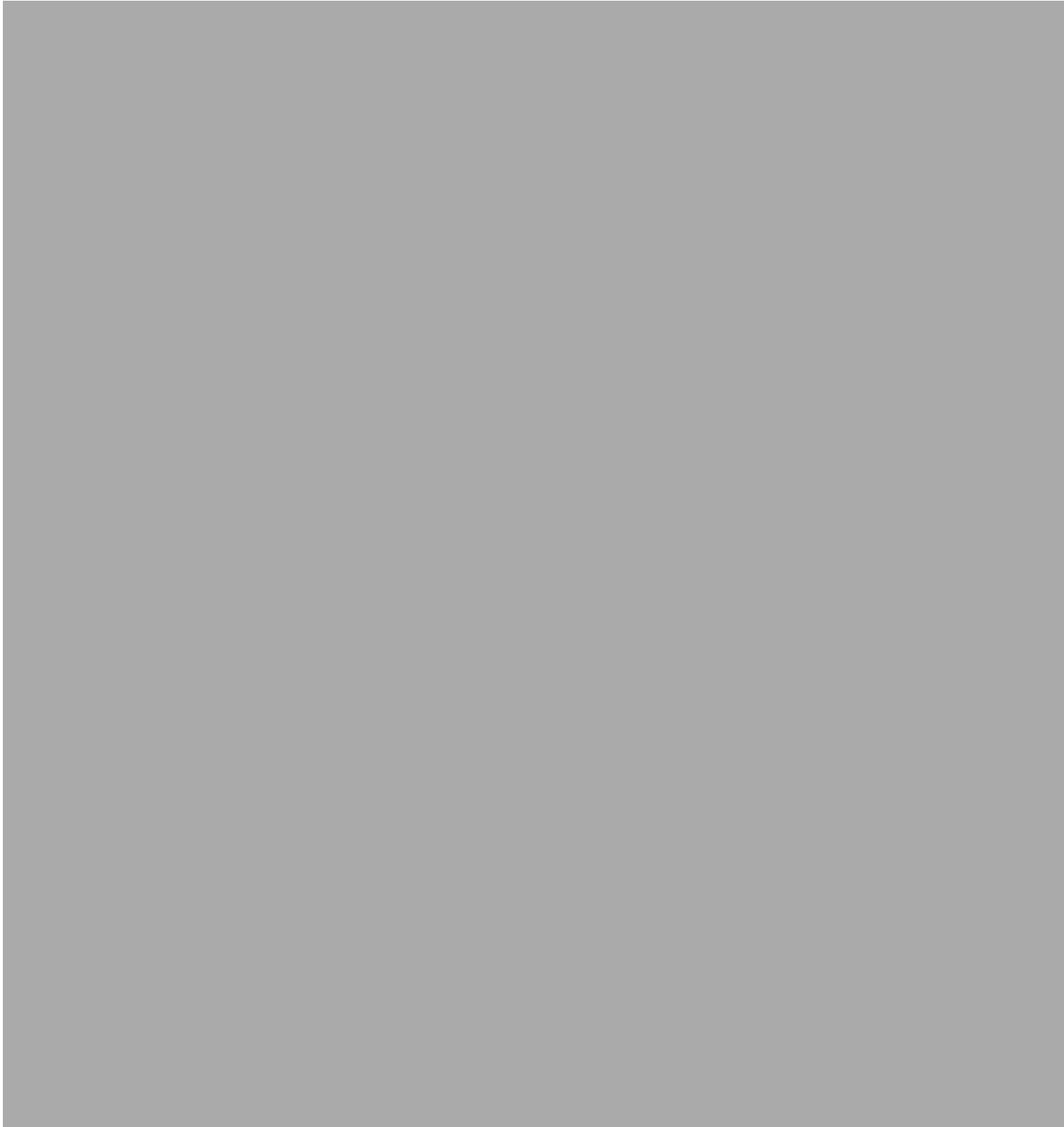
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Understanding and controlling the release mechanism of *Escherichia coli* in double $W_1/O/W_2$ emulsion globules in the presence of NaCl in the W_2 phase†

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Destabilising the structure of double emulsions can be useful for controlling release of substances at the desirable time. Release mechanisms of encapsulated compounds in double emulsions have been described; however, our understanding of bacterial release is limited and the presence of bacteria may affect the emulsion and *vice versa*. In this work, the stability and release properties of double $W_1/O/W_2$ emulsions were studied with or without the presence of GFP-tagged *Escherichia coli* (*E. coli*-GFP) in the inner aqueous phase (W_1) as well as the impact of altering the osmotic balance by adding NaCl in W_2 . Double $W_1/O/W_2$ emulsion preparation and *E. coli*-GFP encapsulation was achieved using a two-step homogenisation process and structure was changed by altering the concentration of hydrophilic surfactant (Tween80, 0.5% to 10%) and W_1 (20% and 40%). The release of *E. coli*-GFP was monitored by culture and observed using fluorescence microscopy. The release of *E. coli*-GFP was significantly ($P < 0.05$) increased when the osmotic balance was altered and the concentration of W_1 was high and Tween80 was low. In contrast, no release of *E. coli*-GFP occurred during osmotic balance alteration when the concentration of W_1 was low and Tween80 was high. Bacterial release occurred due to oil globule bursting independent of diffusion mechanisms. Changing the structure of the emulsion can be used for controlling bacterial release in double emulsions which occurs due to the bursting of the oil globules.




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Modulating the release of *Escherichia coli* in double $W_1/O/W_2$ emulsion globules under hypo-osmotic pressure†

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Double water-in-oil-in-water ($W_1/O/W_2$) emulsions have the potential to encapsulate bacteria and their structure if destabilised can facilitate bacterial release in a time controlled manner. The description of release mechanisms of bacteria in $W_1/O/W_2$ emulsions is limited to hyper-osmotic conditions; however, the mechanism responsible for bacterial release in hypo-osmotic conditions is different and the presence of bacteria may affect the emulsion and *vice versa*. In this work, the stability and release properties of $W_1/O/W_2$ emulsions were studied with or without the presence of GFP-tagged *Escherichia coli* (*E. coli*-GFP) in the inner aqueous phase (W_1). The impact of altering the osmotic balance by diluting the emulsion in a hypo-osmotic solution was also determined. $W_1/O/W_2$ emulsion preparation and *E. coli*-GFP encapsulation was achieved using a two-step homogenisation process and structure was changed by altering the concentration of hydrophilic surfactant (Tween80, 0.5–10%), lipophilic surfactant (PGPR, 1–8%) and W_1 (20% and 40%). The release of *E. coli*-GFP was monitored by culture and observed using fluorescence microscopy. The release of *E. coli*-GFP was significantly ($P < 0.05$) increased when the osmotic balance was altered and at high amounts of W_1 and low concentrations of Tween80 or PGPR. Bacterial release occurred due to oil globule bursting and the viability of bacteria was unaffected by the release mechanism. The concentration of bacteria affected the double emulsion structure during the first step of emulsification but had no effect on emulsion structure during osmotic balance alteration.

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